RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE AND CARBON DIOXIDE FIXATION IN *RHODOMICROBIUM VANNIELII* (RM5) AND *METHYLOCOCCUS CAPSULATUS* (BATH)

by

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CONTENTS

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DECLARATION

This thesis has been composed by myself and has not been used in any previous application for a degree. The results presented here were obtained by myself under the supervision of Dr. C. S. Dow, and all sources of information have been specifically acknowledged by means of reference.

Stephen C. Taylor

SUMMARY

The ribulose 1,5-bisphosphate (RuBP) carboxylase of Rhodomicrobium vannielii (RM5) has been purified and its structure and properties investigated. The enzyme contains both catalytically active large, and regulatory small subunits although the small subunits are shown to be lost under certain conditions. Attention is drawn to the need for care in elucidating RuBP carboxylase quaternary structure, particularly where lengthy purification procedures are involved. The in vivo regulation of carbon dioxide fixation has been examined and found to differ in several respects to that reported for Rhodospirillum rubrum (Slater and Morris, 1973a, b), a physiologically similar organism but whose RuBP carboxylase lacks any small subunits (Tabita and McFadden, 1974a, b).

The use of sucrose density gradient centrifugation in fixed angle rotors for RuBP carboxylase purification has been investigated and is shown to require short centrifugation times giving high resolution whilst allowing relatively large amounts of protein to be used per gradient. The advantages of this technique over gradients in swinging bucket rotors are discussed.

The presence of RuBP carboxylase in extracts of methane grown Methylococcus capsulatus has been demonstrated, this being the first report of this enzyme in a methane oxidising bacterium. The importance of carbon dioxide fixation to M. capsulatus and the possible presence of a functioning Calvin cycle has been investigated by enzyme and radiotracer studies and how RuBP carboxylase activity may be integrated into the overall metabolism of the cell, discussed. The RuBP carboxylase from M. capsulatus (Bath) has been purified and its structure and properties investigated. The enzyme has RuBP oxygenase activity and the metabolism of the product of this reaction, 2-phosphoglycollate, is discussed with respect to its similarity to the serine pathway for one-carbon assimilation. The evolutionary link that M. capsulatus may represent between carbon dioxide and methane assimilating autotrophs is examined.

SECTION I

GENERAL INTRODUCTION

The definition of autotrophy has received considerable attention in recent years veering between that of Schlegel (1975) and Woods and Lascelles (1954), where autotrophs are defined as, 'organisms which are able to grow on inorganic nutrients', and that of Kelly (1971) where, 'autotrophs are organisms which fix carbon dioxide as their prime carbon source via the Calvin cycle (see page 10) and obtain energy from the oxidation of inorganic chemical compounds'. Recently however, Whittenbury and Kelly (1977) have proposed a new definition which broadens the scope of autotrophy such that 'autotrophs are micro-organisms which can synthesise all their cellular constituents from one or more one-carbon (C_1) compounds'. This definition not only encompasses classical autotrophs but also those bacteria characterised as methylotrophs (Colby and Zatman, 1972) and the anaerobic carbon dioxide reducers. Results presented below will give further credence to the Whittenbury and Kelly (1977) definition and consequently, this will be adhered to throughout this thesis. The use of terms such as methylotrophy, will however be retained.

Three groups of autotrophs can be recognised at present, determined by the pathway of C_1 assimilation (i) those with a Calvin cycle, (ii) those with a ribulose monophosphate (RMP) cycle (see page 52) and (iii) those with a serine cycle (see page 60). A number of reviews have appeared on the

Calvin cycle, attention being drawn to those of Kelly (1967; 1971) and McFadden (1973). The RMP cycle and serine pathway have been comprehensively reviewed by Quayle (1972) and Anthony (1975).

The photosynthetic bacterium, Rhodomicrobium vannielii strain RM5 and the methylotroph, Methylococcus capsulatus strain Bath, have been studied in this thesis with a view to understanding the role of carbon dioxide in their metabolism. The General Introduction will therefore review the present knowledge concerning the assimilation of C_1-compounds, particularly carbon dioxide, by micro-organisms. Where relevant, information that has been obtained on higher plant carbon dioxide fixation will also be discussed.

1. GROWTH CHARACTERISTICS OF RHODOMICROBIUM VANNIELII (RM5) AND METHYLOCOCCUS CAPSULATUS (BATH)

a) R. vannielii (RM5)

As with other members of the Rhodospirillaceae, R. vannielii (RM5) is best grown photoheterotrophically with organic compounds serving as photosynthetic electron donors and sources of cellular carbon. A wide range of organic compounds are utilised including dicarboxylic acids, fatty acids and

alcohols. R. vannielii will also grow, albeit slowly, photoautotrophically using sulphide or hydrogen as electron donor and carbon dioxide as principal carbon source (Duchow and Douglas, 1949; Pfennig, 1969). Although R. vannielii requires anaerobic conditions for photosynthesis, the organism will also grow aerobically in the dark.

During photoheterotrophic growth, the balance between oxidation and reduction, is at least in part maintained, either by conversion of organic substrate to carbon dioxide, if it is more oxidised than cell material or by assimilation of carbon dioxide if it is more reduced. Therefore, certain organic substrates require the simultaneous presence of carbon dioxide for their utilisation.

A recent finding has been the ability of Rhodomicrobium and other Rhodospirillaceae to grow on C_1-compounds more reduced than carbon dioxide (Wertlieb and Vishniac, 1967; Yoch and Lindstrom, 1967; Qadri and Hoare, 1968; Pfennig, 1969; Quayle and Pfennig, 1975; Douthit and Pfennig, 1976). Carbon from formate was found to be assimilated as carbon dioxide by a Calvin cycle in Rhodopseudomonas palustris (Yoch and Lindstrom, 1967). Growth of Rhodopseudomonas acidophila (10050) on methanol requires the simultaneous presence of carbon dioxide (1) (Quayle and Pfennig, 1975),

(1) $$2CH_3OH + CO_2 \longrightarrow 3[HCHO] + H_2O$$

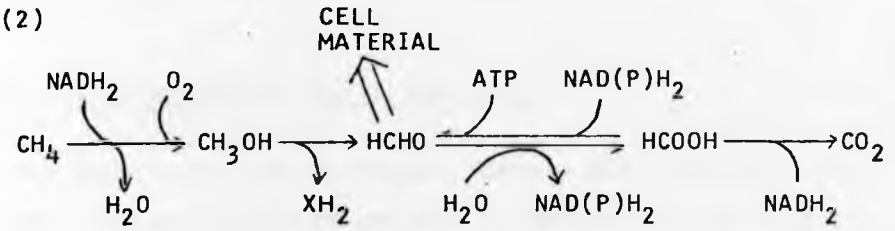
which functions as an 'electron sink' for the excess electrons in methanol. Later work indicated that all carbon entered the cell by way of the Calvin cycle, after complete oxidation of methanol to carbon dioxide (Sahm et al., 1976).

Further details concerning the physiology of Rhodomicrobium are contained in a recent monograph by Whittenbury and Dow (1977).

b) M. capsulatus (Bath)

The facultative thermophile, M. capsulatus (Bath), like all other methane oxidisers, is a strict aerobe due to the requirement of gaseous oxygen for the initial oxidation of methane (Higgins and Quayle, 1970). Methane, methanol and dimethylether are the only substrates shown to support growth of M. capsulatus (Bath). Energy for growth and reproduction, arises from the oxidation of the growth substrate to carbon dioxide. Formaldehyde is an intermediate in this oxidation and it is at this oxidation level that the bulk of carbon is assimilated into cell material (2).

(2)



The enzymes of this oxidation pathway in M. capsulatus (Bath) have been extensively studied by workers at the University of Warwick.

Methylotrophs are generally found to contain a complex internal membrane arrangement and on the basis of this, Whittenbury et al. (1970) divided methylotrophs into two groups. Type I organisms possessed bundles of stacked, disc shaped membrane vesicles whilst Type II organisms possessed layers of paired membranes around the periphery of the cell. Furthermore, the distribution of the two pathways of carbon assimilation in methane oxidisers, parallels the distribution of Type I and II membrane systems. Type I organisms have an RMP cycle and Type II organisms a serine cycle. Although under this classification, M. capsulatus (Bath) is a Type I methylotroph, certain properties of the organism suggest that this may not be so and these anomalies will be discussed in Section 4.

2. ELUCIDATION OF THE CALVIN CYCLE

The techniques used by Benson, Calvin and co-workers for the elucidation of the pathway of carbon dioxide fixation in the green alga *Chlorella* (Calvin, 1962), have become the basis for most work using radioisotopes for tracing metabolic pathways. Because of their importance, a brief summary of these techniques will be given here, together with a description of the Calvin cycle.

Two basic techniques were developed by Calvin, that of rapid sampling following fixation of [^{14}C]-bicarbonate and identification of radiolabelled compounds and secondly the use of ^{14}C saturation experiments. By measuring the amount of isotope present in various compounds at early times after fixation of [^{14}C]-bicarbonate, a series of curves of varying slopes and shapes were obtained. Curves for the amount of isotope present in compounds serving as ports of entry for the labelled substrate have negative slopes and for those compounds derived from them, positive slopes. It was then possible to identify many intermediates of the Calvin cycle and also the products of carboxylation. It was also found that most compounds became quickly saturated with radioactivity and yet the amount of these compounds present is always small and unfluctuating. The active pool sizes of these compounds could then be measured and by

changing an external variable, alterations in pool sizes could be followed. It was found with Chlorella that when deprived of light there was a transient rise in the pool size of 3-phosphoglycerate and a corresponding fall in the pool size of ribulose 1,5-bisphosphate (RuBP) (Bassham et al., 1954; Bassham and Kirk, 1960). This corresponded with 3-phosphoglycerate being the product of carboxylation, previously suggested by a negative slope of incorporation of label into this compound, and RuBP being the acceptor compound for carbon dioxide. The removal of light effectively blocked the energy dependent conversion of 3-phosphoglycerate to triose phosphates without decreasing the rate of formation of 3-phosphoglycerate from RuBP. Similar experiments limiting the availability of carbon dioxide (Wilson and Calvin, 1955), led to the accumulation of ribulose and a fall in the concentration of 3-phosphoglycerate.

Together with studies of enzyme activities in cell-free extracts, the cycle of carbon dioxide fixation was elucidated. The basic cycle is shown in Figure 1a. A possible alternative to the rearrangement sequence shown, is also indicated (Fig. 1b). The first key enzyme of the cycle is RuBP carboxylase which catalyses the carboxylation of RuBP to give 3-phosphoglycerate (3).

Fig. 1a The Calvin cycle
(after Quayle and Ferenci, 1978)

Abbreviations:

RuBP, ribulose 1,5-bisphosphate;

PGA, 3-phosphoglycerate;

DPGA, 1,3-diphosphoglycerate;

GAP, glyceraldehyde 3-phosphate;

DHAP, dihydroxyacetone phosphate;

FBP, fructose 1,6-bisphosphate;

FMP, fructose 6-phosphate;

EMP, erythrose 4-phosphate;

XuMP, xylulose 5-phosphate;

SBP, sedoheptulose 1,7-bisphosphate;

SMP, sedoheptulose 7-phosphate;

R5P, ribose 5-phosphate;

Ru5P, ribulose 5-phosphate

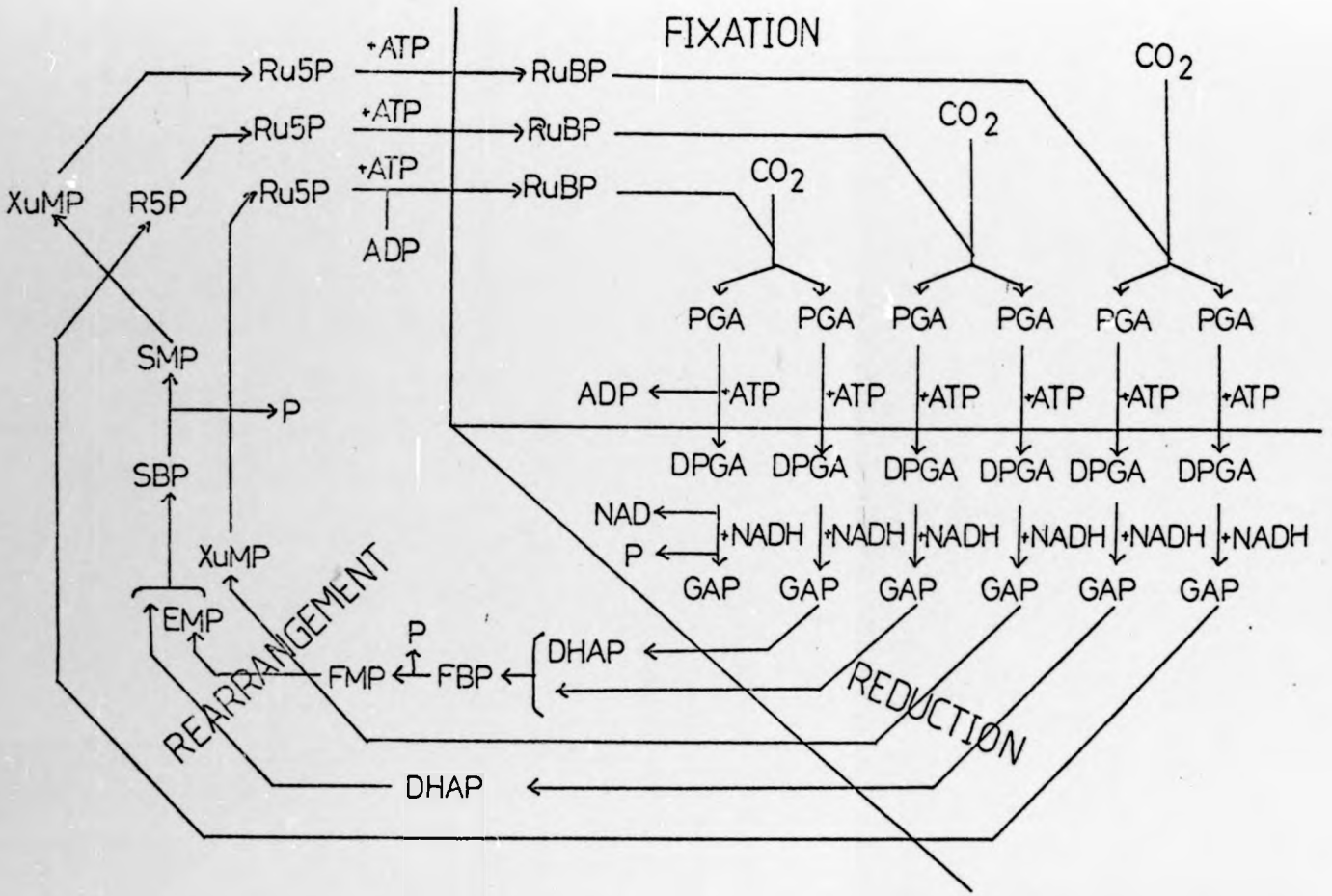
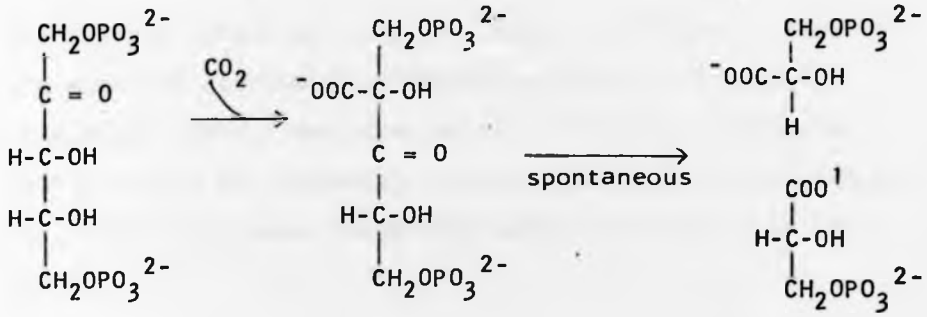

FIXATION
Ru5P
+ATP
RuBP
CO2
XuMP
R5P
ADP
PGA
DPGA
NAD
+NADH
P
GAP
SMP
SBP
EMP
XuMP
REARRANGEMENT
FMP
FBP
DHAP
REDUCTION

Fig. 1b Hypothetical variant of the Calvin cycle (after Quayle and Ferenci, 1978)

in cycle

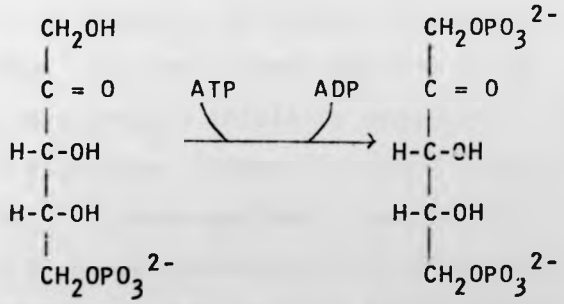
FIXATION
REDUCTION
REARRANGEMENT
CO2
CO2
CO2
RuBP
RuBP
RuBP
Ru5P
Ru5P
Ru5P
+ATP
+ATP
+ATP
ADP
PGA
PGA
PGA
PGA
PGA
PGA
+ATP
+ATP
+ATP
+ATP
+ATP
+ATP
DPGA
DPGA
DPGA
DPGA
DPGA
DPGA
+NADH
+NADH
+NADH
+NADH
+NADH
+NADH
GAP
GAP
GAP
GAP
GAP
GAP
NAD
P
DHAP
DHAP
FBP
FBP
P
FMP
FMP
EMP
GAP
SMP
XuMP
XuMP

(3)



The rest of the cycle functions to regenerate RuBP and to give the net synthesis of one mole of triose phosphate from three moles of carbon dioxide. This entails the phosphorylation and reduction of 3-phosphoglycerate followed by a rearrangement sequence of which the final step is catalysed by the second key enzyme of the cycle, phosphoribulokinase. This involves the generation of RuBP from ribulose,5-phosphate (Ru5P) (4).

(4)



The overall scheme of reactions shown in Figure 1a requires six moles of reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$) and nine moles of adenine triphosphate (ATP) for the net synthesis of one mole of triose phosphate (5). This indicates the highly endergonic nature of the cycle.

(5) $3CO_2 + 6NADPH_2 + 9ATP \longrightarrow$ glyceraldehyde 3-phosphate + $6NADP + 9ADP + 9P_i$

The presence of RuBP carboxylase and phosphoribulokinase in cell-free extracts, is generally considered as an indication of the operation of the Calvin cycle.

Before considering the occurrence of the Calvin cycle and other pathways of carbon dioxide fixation in bacteria, mention should be made of a postulated second pathway of carbon dioxide fixation in certain higher plants (Hatch and Slack, 1966). This was based upon the finding that label from [^{14}C]-bicarbonate initially appeared in the C_4 acids, malate and aspartate. Plants in which this occurs exhibit a higher rate of photosynthesis than the so called C_3-plants, which have 3-phosphoglycerate as primary fixation product. However, as discussed by Zelitch (1975), carboxylation yielding malate and aspartate represents a mechanism for

increasing the concentration of carbon dioxide at the site of action of RuBP carboxylase and not a net fixation pathway.

3. PATHWAYS OF CARBON DIOXIDE FIXATION IN BACTERIA

a) The reductive carboxylic acid cycle

Considerable controversy has followed the suggestion made by Evans et al. (1966), of a cycle of carbon dioxide fixation termed the reductive carboxylic acid cycle. This cycle was reported in the photosynthetic bacterium, Chlorobium thiosul atophilum, when grown anaerobically in the light with carbon dioxide as sole source of carbon. It is effectively a reversal of the tricarboxylic acid cycle and results in the net synthesis of one mole of oxaloacetate from four moles of carbon dioxide with the regeneration of acetyl CoA, the primary carbon dioxide acceptor (Fig. 2). Two novel carboxylation reactions are involved, catalysed by pyruvate synthase (6) and α-oxoglutarate synthase (7). These enzymes have been found in cell-free extracts of C. thiosulphatophilum (Evans et al., 1966), Rhodospirillum rubrum (Buchanan et al., 1967), Chromatium D (Buchanan and Arnon, 1965) and the methanogenic bacterium Methanobacterium thermoautotrophicum (Zeikus et al., 1977). Evidence for the

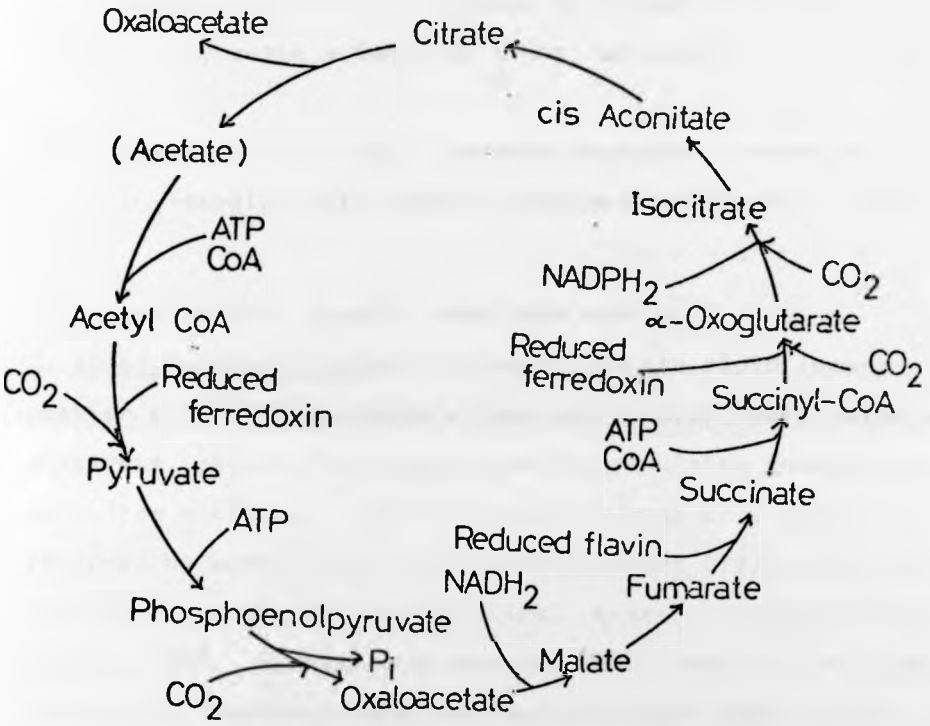


Fig. 2 The reductive carboxylic acid cycle (after Evans et al., 1966)

(6) Acetyl CoA + CO_2 + reduced ferredoxin ⟶
pyruvate + CoA + oxidised ferredoxin

(7) Succinyl CoA + CO_2 + reduced ferredoxin ⟶
α-oxoglutarate + CoA + oxidised ferredoxin

cycle has however largely come from work with C. thiosulphatophilum and is based upon the rapid incorporation of [^{14}C]-bicarbonate into amino acids, particularly glutamate, and on the presence of the requisite enzymes in cell-free extracts. This evidence has been critically reviewed by both Quayle (1972) and McFadden (1973) who have indicated a number of deficiencies. Kinetic studies (Evans et al., 1966; Sirevag and Ormerod, 1970) have not included very early fixation times and have also been done at non-physiological carbon dioxide concentrations. Furthermore, several enzymes notably citrate lyase, have insufficient activity in cell-free extracts, to account for the in vivo carbon dioxide fixation rate. The failure (Buchanan et al., 1972; Buchanan and Sirevag, 1976) to detect RuBP carboxylase in C. thiosulphatophilum was also taken to suggest a reductive carboxylic acid cycle. However, this enzyme has been detected by Smillie et al. (1962) and Tabita et al. (1974a) although at a low specific activity

More recently, studies with a carbon isotope fractionation technique (Sirevag *et al.*, 1977), which is based upon the preferential use of ^{12}C rather than ^{13}C by RuBP carboxylase as opposed to the non-selectivity of phosphoenolpyruvate (PEP) carboxylase, have shown that the contribution of RuBP carboxylase to growth of *Chlorobium* on carbon dioxide is less than that for *Chromatium*, *R. rubrum* or *Chlamydomonas*.

In conclusion therefore, there is as yet, no evidence to suggest that the reductive carboxylic acid cycle functions as a full cycle for carbon dioxide fixation in any organism. However, certain bacteria do have the enzymic capability to support part of the cycle which in *Chlorobium* and more especially *M. thermoautotrophicum* (Fuchs and Stupperich, 1978), may have an important role.

b) *The Calvin cycle*

Most of the work on bacterial carbon dioxide fixation has been done with photosynthetic bacteria notably the *Rhodospirillaceae*. As previously indicated, although able to grow photoautotrophically, these organisms exhibit higher growth rates under photoheterotrophic conditions where carbon dioxide fixation assumes far less importance.

The kinetic studies of Stoppani et al. (1955) with Rhodopseudomonas capsulatus, established that carbon dioxide is fixed mainly via a Calvin cycle although fixation into malate, presumably due to C_3-carboxylation reactions, was also evident. Under conditions of non-photosynthetic growth, carbon dioxide fixation by the Calvin cycle was of less significance.

Contradictory results have been obtained with R. palustris. Yoch and Lindstrom (1967) reported that photoautotrophically grown R. palustris assimilated both carbon dioxide and formate (which is assimilated as carbon dioxide and hydrogen), primarily into glutamate and amino acids after a fixation time of 15s and phosphate esters accounted for less than 6% of the fixed radioactivity. These results were suggestive of a reductive carboxylic acid cycle. However, the labelling pattern from carbon dioxide in photoheterotrophically grown cells, was consistent with the operation of a Calvin cycle. In contrast are the results of Stokes and Hoare (1969), also for photoautotrophically grown R. palustris. With both [^{14}C]-formate and [^{14}C]-bicarbonate, phosphate esters were the first assimilation products and the key enzymes of the Calvin cycle were present in cell-free extracts. The different results obtained by Stokes and Hoare (1969) to those of Yoch and Lindstrom (1967), may be due to differences in bacterial strains, growth and experimental conditions.

Rhodospirillum rubrum has been the subject of several investigations into the mechanism of carbon dioxide fixation. Glover et al. (1952), first reported that [^{14}C]-bicarbonate was initially assimilated, by photoheterotrophically grown R. rubrum, into 3-phosphoglycerate when incubated in the presence of hydrogen and carbon dioxide (i.e. autotrophically). Similar results were obtained by Hoare (1963) with R. rubrum grown on malate. However, both of these studies used photoheterotrophically grown cells for measuring carbon dioxide fixation autotrophically. Anderson and Fuller (1967a) using autotrophically grown R. rubrum reported a negative slope of incorporation into phosphate esters with, after 1s incubation, 75% of the radioactivity on the chromatogram in a 3-phosphoglycerate. This clearly indicates that under photoautotrophic conditions, the Calvin cycle is the major assimilatory pathway for carbon. However, under photoheterotrophic conditions following growth on malate, carbon dioxide fixation was of far less importance and Calvin cycle activity was very much reduced. (Anderson and Fuller, 1967b). Glycollate was the first stable radioactive product although the mechanism of its labelling was not elucidated. Slater and Morris (1973a, b) again using R. rubrum, found the Calvin cycle to be the major pathway of carbon dioxide assimilation. Growth conditions were also shown to profoundly effect the pattern of uptake of labelled carbon dioxide. Similarly, light intensity was found by

Porter and Merrett (1972) to effect both RuBP carboxylase activity and the uptake of carbon dioxide in R. rubrum, with both parameters having higher activity in cells grown at high light intensity (9000 lux) as opposed to low light intensity (1500 lux).

With most other photosynthetic prokaryotes, the Calvin cycle has been implicated where [^{14}C]-carbon dioxide uptake experiments have been done. Autotrophically grown Chromatium D assimilated carbon dioxide primarily into 3-phosphoglycerate and aspartate, indicating the activity of RuBP carboxylase and C_3-carboxylases (Fuller et al., 1961). Similarly, although Pelroy and Bassham (1972) clearly demonstrated activity of the Calvin cycle in a number of cyanobacteria, carboxylation of phosphoenolpyruvate has also been shown to account for a significant proportion of the total carbon dioxide fixed by Anacystis nidulans (Jansz and Maclean, 1973). In the photosynthetic halophile, Halobacterium halobium, carbon dioxide is assimilated into acid stable products, indicative of the Calvin cycle (Danon and Caplan, 1977).

Within the non-photosynthetic bacteria, the Calvin cycle has been shown to be the major pathway of autotrophic carbon dioxide fixation in species of the hydrogen bacteria (Bergmann et al., 1958), Pseudomonas facilis (McFadden, 1959), thiobacilli (Din et al., 1967; McCarthy and Charles, 1974) and the

nitrifying bacteria (Aleem, 1965). The facultative hydrogen bacterium, Paracoccus denitrificans contains both RuBP carboxylase and phosphoribulokinase when grown autotrophically on methanol or hydrogen and carbon dioxide although these enzymes are not detectable in cells grown heterotrophically on acetate or glycollate (Kornberg et al., 1960; Cox and Quayle, 1975). During growth of Pseudomonas oxalaticus on formate, carbon is assimilated via the Calvin cycle (Quayle, 1961; Dijkhuizen et al., 1977) although RuBP carboxylase is undetectable in extracts of heterotrophically grown cells (Quayle and Keech, 1960). Recently, the Calvin cycle has been implicated in the metabolism of Desulfovibrio vulgaris with the finding that cell-free extracts show RuBP dependent incorporation of [^{14}C]-carbon dioxide into acid stable products (Alvarez and Barton, 1977).

In conclusion, the Calvin cycle is the only proven indispensible mechanism of autotrophic carbon dioxide fixation. The situation during the heterotrophic growth of facultative autotrophs is complex although there is little doubt that carbon dioxide fixation under such conditions does not contribute greatly to net carbon assimilation and consequently, C_3-carboxylation assumes relatively more importance.

4. RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE

a) Introduction

The carboxylation of RuBP is a reaction of central importance in the Calvin cycle for carbon dioxide fixation. Because of its key role in this cycle and its ubiquity amongst carbon dioxide fixing autotrophs, the enzyme has been extensively studied for three decades.

Early studies were concerned with higher plant and algal RuBP carboxylase where the enzyme may account for up to 50% (w/w) of the total soluble protein. However, the diversity of RuBP carboxylase structure only became apparent with examination of the enzyme from prokaryotic sources where it accounts for a much smaller proportion of the soluble protein.

A division of RuBP carboxylases into three classes on the basis of molecular weight, has been proposed by Anderson et al. (1968). These classes are small (mol. wt. 80-120,000), intermediate (mol. wt. 240-360,000) and large (mol. wt. approximately 500,000) and despite differences in molecular weight, enzymes from all classes have the same catalytic activity.

Inclusions, termed 'polyhedral bodies', have been observed in cyanobacteria (Gantt and Conti, 1969; Wolk, 1973), nitrifying bacteria (Murray and Watson, 1965; Pope et al.,

1969; Watson and Waterbury, 1971; Wullenweber *et al.*, 1977), thiobacilli (Shively *et al.*, 1970) and possibly in *R. vannielii* (RM5) (France, 1978). These structures range in diameter from 90 to 500 nm and number up to 80 per cell. They were first isolated by Shively *et al.* (1973a), from *Thiobacillus neapolitanus* and shown to be surrounded by a monolayer envelope. RuBP carboxylase was also found to be associated with these bodies and consequently the name 'carboxysomes' was proposed. Further studies indicated that these carboxysomes contained paracrystalline arrays of RuBP carboxylase (Shively *et al.*, 1973b). With the techniques developed by Shively, carboxysomes have since been characterised from *Thiobacillus intermedius* (Purohit *et al.*, 1976) and *Anabaena cylindrica* (Codd and Stewart, 1976) although they have also been found to be very unstable in some organisms. Whether carboxysomes function in carbon dioxide fixation or are involved in enzyme storage (Shively, 1974) awaits answering and as of yet, little evidence to suggest either role has been accrued. However, in view of their widespread occurence amongst autotrophs, they may well have a significant role in the regulation of the Calvin cycle.

b) Protein chemistry

As indicated above, three broad classes of RuBP carboxylase are recognised although the enzyme from all eukaryotic sources studied, is of the large molecular weight class. The protein can be dissociated into subunits by treatment with sodium dodecyl sulphate (SDS) (Rutner and Lane, 1967) or urea (Sugiyama and Akazawa, 1970). RuBP carboxylase from all eukaryotic organisms dissociates into two types of subunit as revealed by SDS-polyacrylamide gel electrophoresis: large (L) polypeptide subunits (mol. wt. 50-58,000) and small (S) polypeptide subunits (mol. wt. 11-18,000). It has been established that equal numbers of these subunits are present, such that the overall quaternary structure of the enzyme from green plants and unicellular algae (mol. wt. approximately 500-550,000) consists of eight large plus eight small subunits (L_8S_8). Although there is uncertainty regarding the spatial arrangement of the subunits, the work of Baker *et al.* (1975, 1977) with optical diffraction and electron microscopy, suggests that the large subunits form a cube with the small subunits attached to the outside of this cube.

Within the prokaryotes there is both more diversity and uncertainty as to the size and structure of RuBP carboxylases. Table 1 contains a compilation of known molecular weights and quaternary structures for RuBP carboxylases isolated from all

major classes of prokaryotes and eukaryotes. Where contradictory results have been published, all sets of values have been given.

Enzymes from the cyanobacteria, the hydrogen bacteria, Chromatium D, E. halophila and certain thiobacilli are of the large molecular weight class. Those from C. thiosulphatophilum and T. denitrificans are of intermediate size and that of R. rubrum is the only known small molecular weight RuBP carboxylase.

Considerable doubt surrounds the nature of the enzyme in the Rhodopseudomonads. By density gradient centrifugation of impure RuBP carboxylase, Anderson et al. (1968) reported sedimentation coefficients for the R. spheroides and R. palustris enzymes, relative to the R. rubrum enzyme, of 14.5S and 12S corresponding to approximate molecular weights of 360,000. However, it has recently been reported that apparently low sedimentation coefficients are obtained for the RuBP carboxylases of Paracoccus denitrificans (Bowien, 1977) and Alcaligenes eutropha (Bowien and Mayer, 1978), both of which have high molecular weights of approximately 550,000. Consequently, the correlation between relative sedimentation coefficient and molecular weight, used by Anderson et al. (1968), may be invalid. Akazawa et al. (1970) reported a molecular weight of the R. spheroides RuBP carboxylase of 240,000 using

Table 1 Comparisons of RuBP carboxylase from autotrophs

Enzyme Source	molecular wt or $S_{20,w}$	quaternary structure	inhibition by 1mM 6-phosphogluconate	oxygenase activity
Photosynthetic bacteria				
Rhodospirillum rubrum				
(Tabita and McFadden, 1974a, b)	114,000	L_2	no	yes
(Akazawa et al., 1970)	83,000	ND	ND	ND
Chlorobium thiosulphatophilum				
(Tabita et al., 1974a)	360,000	L_6	no	ND
Rhodopseudomonas spheroides				
(Anderson et al., 1968)	14.5S approx 360,000	ND	ND	ND
(Akazawa et al., 1970)	240,000	ND	ND	ND
(Gibson and Tabita, 1977a)	360,000	L_6	no	ND
	550,000	L_8S_8	yes	ND

Enzyme Source	molecular wt or $S_{20,w}$	quaternary structure	inhibition by 1mM 6-phosphogluconate	oxygenase activity
Rhodopseudomonas palustris (Anderson et al., 1968)	12S	ND	ND	ND
Rhodopseudomonas capsulata (Gibson and Tabita, 1977b)	ND	ND	yes	ND
	ND	ND	no	ND
Chromatium D (Akazawa et al., 1972)	550,000	L_8S_8	yes	yes
Ectothiorhodospira halophila (Tabita and McFadden, 1976)	600,000	L_8S_8	yes	ND
Chemosynthetic bacteria				
Thiobacillus denitrificans (McFadden and Denend, 1972)	350,000	ND	no	ND

Enzyme Source	molecular wt or $S_{20,w}$	quaternary structure	inhibition by 1mM 6-phosphogluconate	oxygenase activity
Thiobacillus intermedius (Purohit et al., 1976)	455,000	L_8	yes	ND
Thiobacillus novellus (McCarthy and Charles, 1975)	498,000	ND	yes	ND
Thiobacillus A2 (Charles and White, 1976a, b)	521,000	L_8S_8	yes	ND
Alcaligenes eutropha (Purohit and McFadden, 1977)	516,000	L_8S_8	yes	yes
Pseudomonas oxalaticus (McFadden, 1977)	360,000	L_6S_6	ND	ND
Paracoccus denitrificans (Bowien, 1977)	513,000	L_8S_8	yes	ND

Enzyme Source	molecular wt or $S_{20,w}$	quaternary structure	inhibition by 1mM 6-phosphogluconate	oxygenase activity
Cyanobacteria				
Agmenellum quadruplicatum (Tabita et al., 1974b)	456,000	L_8	ND	ND
Anabaena cylindrica (Tabita et al., 1976)	452,000	L_8	ND	ND
Anabaena variablis (Takabe et al., 1976)	18S	L_8S_8	ND	ND
Plectonema boryanum (Takabe et al., 1976)	18S	L_8S_8	ND	ND
Aphanocapsa (Codd and Stewart, 1977)	525,000	L_8S_8	yes	yes

Enzyme Source	molecular wt or $S_{20,w}$	quaternary structure	inhibition by 1mM 6-phosphogluconate	oxygenase activity
Green Algae				
Euglena gracilis (McFadden et al., 1975)	525,000	L_8S_8	yes	yes
Chlamydomonas reinhardi (Givan and Criddle, 1972)	530,000	L_8S_8	ND	ND
Chlorella fusca (Lord and Brown, 1975)	530,000	L_8S_8	yes	yes
Higher Plants				
Spinach (Kawashima and Wildman, 1970)	560,000	L_8S_8	yes	yes
spinach-beet (Kawashima and Wildman, 1970)	560,000	L_8S_8	ND	ND

Enzyme Source	molecular wt or $S_{20,w}$	quaternary structure	inhibition by 1mM 6-phosphogluconate	oxygenase activity
tobacco (Kawashima and Wildman, 1970)	525,000	L_8S_8	ND	yes
French bean (Kawashima and Wildman, 1970)	17.95	L_8S_8	ND	ND

L = large subunit, 50-58,000 mol.wt.

S = small subunit, 11-18,000 mol.wt.

ND = not determined

a gel filtration method. More recently however, Gibson and Tabita (1977a, b) have indicated the presence of two forms of RuBP carboxylase, both in R. spheroides and in R. capsulata with, from the former source, molecular weights of 550,000 and 360,000. These two forms of enzyme have quaternary structures of L_8S_8 and L_6 respectively, and antiserum prepared against the L_6 form did not cross-react with the L_8S_8 form.

RuBP carboxylases lacking small subunits have also been reported for Agmenellum quadruplicatum (Tabita et al., 1974) and Anabaena cylindrica (Tabita et al., 1976). However, the L_8 quaternary structure assigned to these enzymes has been questioned (Codd and Stewart, 1977) following work with the Aphanocapsa RuBP carboxylase. It was found that the small subunits were lost from this enzyme on acid precipitation, a purification technique widely used by Tabita and co-workers. Similar loss of the small subunits, has been reported by McFadden and Tabita (1974), for the E. halophila RuBP carboxylase and by Shively (personal communication), for the enzyme from I. neopolitanus on ammonium sulphate treatment.

It is therefore clear that the quaternary structure of many RuBP carboxylases is unstable. The widespread use of ammonium sulphate precipitation, acid precipitation and heat treatment for purification of this enzyme provides an

explanation for the variable results that have been obtained in connection with its structure. The non cross-reaction by antiserum between the L_6 and L_8S_8 forms of R. spheroides and R. capsulata enzymes (Gibson and Tabita, 1977a, b) would be an expected result if the small subunits surround the large subunits in the L_8S_8 form, and so mask the antigenic determinants. Indeed, as will be shown later, the amino acid composition of large subunits from diverse organisms is always very similar and if the large subunits in the L_8S_8 form were exposed, cross-reaction would almost certainly occur even if the L_6 and L_8S_8 forms represented two totally separate enzymes. Confirmation of the existence of two types of RuBP carboxylase in these organisms, awaits further immunological analysis with antisera directed against isolated subunits.

The dimeric enzyme of R. rubrum is the smallest form of RuBP carboxylase characterised to date. There is however, preliminary evidence that the enzyme of Thiocapsa may be functional in a monomeric form (McFadden, 1977).

In conclusion, although RuBP carboxylases from different sources may clearly have very different molecular weights, the existence of L_2, L_6 and L_8 quaternary structures is in doubt.

No complete amino acid sequences are available for either large or small subunits from any RuBP carboxylase. Comparison of enzymes therefore, has to be based upon amino acid composition. Using the statistical method of Marchalonis and Weltman (1971) for such data, it was found that the large subunit is structurally homologous among divergent autotrophs ranging from photosynthetic bacteria to higher plants (McFadden, 1975; Takabe and Akazawa, 1975) indicating its genetic conservation during evolution. In contrast, the small subunit was found to be structurally quite different among divergent autotrophs. Indeed, the RuBP carboxylase from five different species of tobacco was shown to have small subunits with significantly different amino acid composition (Kawashima et al., 1971; Kawashima and Wildman, 1971).

Immunological studies have also indicated the homologous nature of large subunits from different sources (McFadden, 1973). Antiserum against eukaryotic RuBP carboxylases was shown to cross-react with RuBP carboxylase from several cyanobacteria (Lord et al., 1975; Takabe et al., 1976). The small subunit however, will generally only cross-react with antiserum raised against itself (Gray and Kekwick, 1974; Takabe and Akazawa, 1975). Of interest was the finding that antiserum to the R. rubrum RuBP carboxylase would not cross-

react with RuBP carboxylase from any of a number of sources tested (Tabita and McFadden, 1974b). Whether this is due to antigenic sites on the large subunits of other carboxylases being masked by the small subunits or to the R. rubrum enzyme being only distantly related to other RuBP carboxylases, awaits clarification.

When examined by isoelectric focussing, the subunits of RuBP carboxylase were found to be heterogeneous (Kung et al., 1974). The large subunit appears to have three variants whilst the small subunit has any number from one to four. Heterogeneity has also been observed for the large subunit of the A. eutropha RuBP carboxylase (Purohit and McFadden, 1976, 1977). In this instance, the variants have different molecular weights (52,000; 56,000) and could be separated by SDS-polyacrylamide gel electrophoresis (PAGE) using a low protein load per gel. Whether these variants are products of different genes or arise as a result of modification of a single gene product is unclear.

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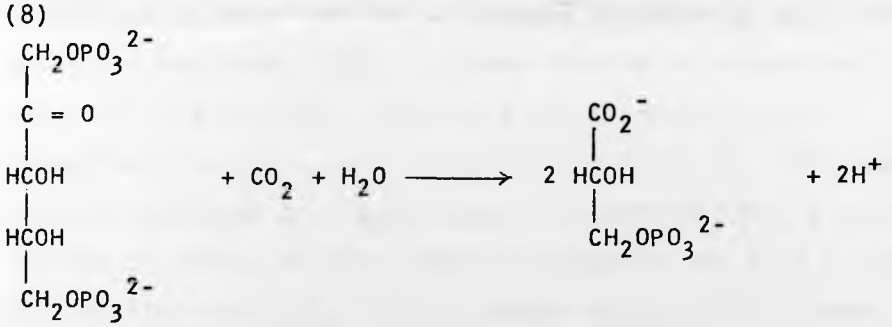
c) Catalytic activity

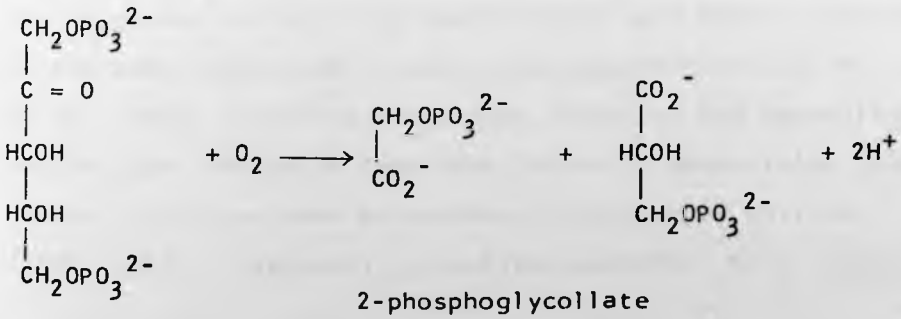
RuBP carboxylase (3-phospho-D-glycerate carboxylase [dimerizing], EC 4.1.1.39) has two enzyme activities associated with it. In addition to catalysing carboxylation of RuBP to produce two molecules of 3-phosphoglycerate (reaction 8), this enzyme also catalyses the oxygenation of RuBP to produce a molecule each of 3-phosphoglycerate and 2-phosphoglycollate (reaction 9) (Bowes et al., 1971).

(8)



RuBP 3-phosphoglycerate

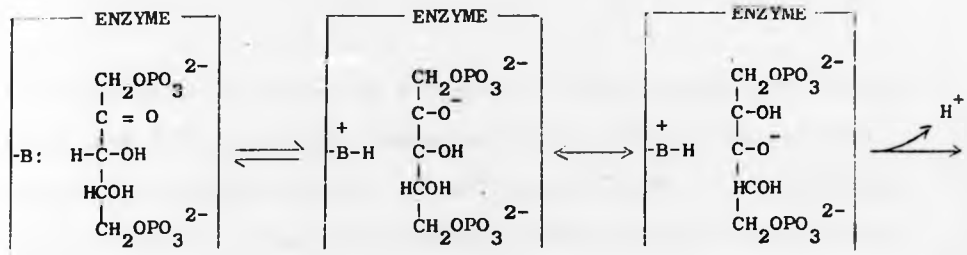
(9)



2-phosphoglycollate

This oxygenase activity has been found to be associated with all RuBP carboxylases investigated to date (see Table 1). Recently, Branden (1978) working with parsley RuBP carboxylase has reported that the carboxylase and oxygenase activities are in fact different enzymes, separable at high pH. Whether this work can be substantiated requires further investigation.

The essential mechanistic details of the reaction were worked out by Rose and his colleagues (Fiedler et al., 1967; Mullhofer and Rose, 1965). Carbon dioxide or oxygen addition occurs at C-2 of RuBP, this being facilitated by prior formation of a 2,3-enediol intermediate (Fig. 3). Although there is evidence to suggest that bicarbonate (HCO_3^-) is the species of carbon dioxide taken up by whole cells of R. rubrum (Christeller and Laing, 1978), Cooper et al. (1969) have shown that CO_2 is the active species in the RuBP carboxylase reaction. The carboxylase activity provides the carbon fixing step in the Calvin cycle. The role and importance of the oxygenase activity has however been more open to question. It has been implicated in plant photorespiration (Lorimer et al., 1973) involving glycollate formation and metabolism and as such, may be an important factor in determining crop yields. This has been extensively discussed by Zelitch (1973, 1975). Similarly, glycollate excreted by R. rubrum



tautomeric forms of enediol intermediate

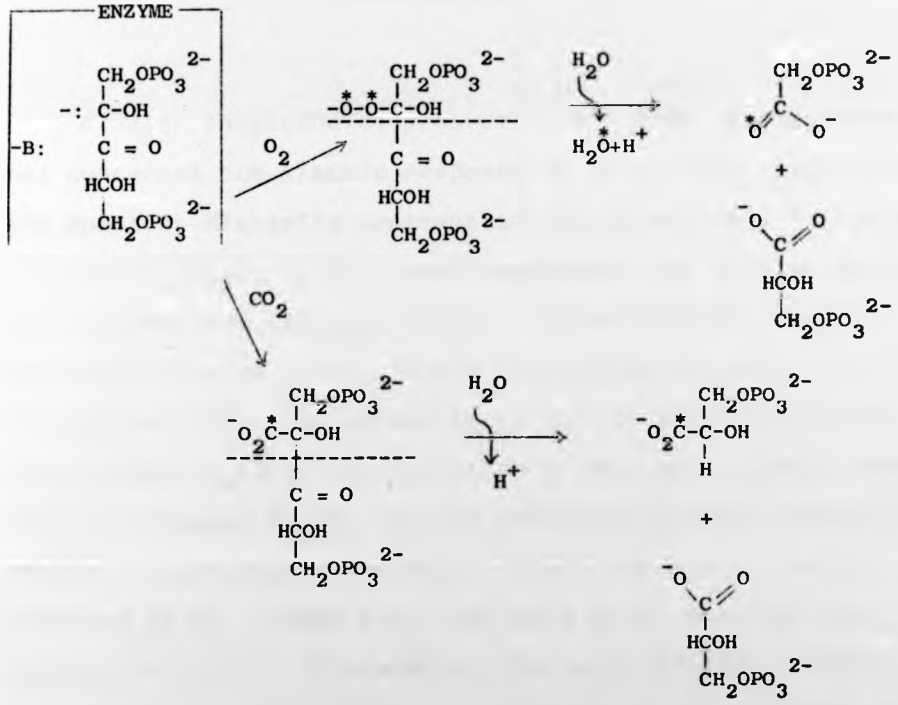


Figure 3 Proposed mechanism of cleavage of RuBP by RuBP carboxylase-oxygenase

is considered to arise as a result of RuBP oxygenase activity (Codd and Smith, 1974). Because of the involvement of an oxygen sensitive, enolate anion form of RuBP in the carboxylation reaction, the production of phosphoglycollate appears to be an unavoidable consequence of this enzyme operating in an aerobic environment (Lorimer and Andrews, 1973).

A major longstanding problem of all RuBP carboxylases has concerned the kinetic response to substrates. Specifically, the apparent Michaelis constant of the carboxylase for carbon dioxide ($K_m(CO_2)$) *in vitro* was considered too high to account for the observed *in vivo* rates of carbon dioxide fixation. The concentration of CO_2 in air equilibrated aqueous solutions is 10 μM at 25°C, corresponding to 0.6 mM bicarbonate and the *in vitro* $K_m(CO_2)$ was more than a magnitude greater than this. Decreased $K_m(CO_2)$ values were obtained by assaying the enzyme at high magnesium (Mg^{2+}; 20-45 mM) concentrations (Bassham *et al.*, 1968) but these were still too high for *in vivo* activity. Furthermore, the order of addition of reagents to the RuBP carboxylase assay affects the time course of the subsequent reaction and for maximal activity, enzyme must be incubated with Mg^{2+} and CO_2 prior to initiation of the reaction with RuBP (Kuehn and McFadden, 1969; Tabita and McFadden, 1974a; Lorimer *et al.*, 1976).

Reports of a form of carboxylase with a sufficiently low $K_m(CO_2)$ to account for rates of carbon dioxide fixation in plants (Bahr and Jensen, 1974; Badger and Andrews, 1974) were shown to be due to an induced activation of the enzyme by appropriate physical conditions and concentrations of CO_2 and Mg^{2+} (Laing et al., 1975; Andrews et al., 1975; Lorimer et al., 1977). The carboxylase is activated and stabilized in an active form by preincubation with Mg^{2+} and CO_2. During preincubation, the enzyme reacts with CO_2 (rather than HCO_3^-) and Mg^{2+} in a reversible reaction to give an active equilibrium complex of enzyme-CO_2-Mg^{2+}. The activity of this complex is dependent upon concentrations of CO_2 and Mg^{2+} and upon the preincubation pH (Lorimer et al., 1976). Whether the CO_2 molecule that enters into complex formation is the same CO_2 molecule that carboxylates RuBP, is unknown. The presence of RuBP during preincubation appears to force the enzyme into a form that is less catalytically active. A similar activation of the enzyme is required for RuBP oxygenase activity (Badger and Lorimer, 1976). Failure to fully activate the enzyme appears to be the principal cause of the wide variation in reported values for $K_m(CO_2)$.

RuBP carboxylase activity is highly dependent upon added Mg^{2+} (Kawashima and Wildman, 1970). Other divalent cations, notably Ni^{2+} and Co^{2+}, may substitute for Mg^{2+}

with the plant enzyme, but the efficacy depends upon the age and purity of the enzyme preparation. The enzyme of R. rubrum is specific for Mg^{2+} (Tabita and McFadden, 1974a).

A number of compounds have been reported to influence the activity of RuBP carboxylase, the most important of which is 6-phosphogluconate (6PG) (Chu and Bassham, 1972, 1973; Tabita and McFadden, 1972). Inhibition of activity can be caused by 6PG with a Ki of about 0.1 mM (Tabita and McFadden, 1972). It is only the large molecular weight class of RuBP carboxylases that are inhibited with the enzymes of R. rubrum (Tabita and McFadden, 1974b), C. thiosulphatophilum (Tabita et al., 1974), T. denitrificans (McFadden and Denend, 1972) and the low molecular weight form from R. spheroides (Gibson and Tabita, 1977a), being unaffected. However, Chu and Bassham (1973) found with the spinach enzyme that 6PG could also activate the carboxylase if it was added prior to the addition of RuBP during preincubation. This they interpreted as 6PG preventing the inhibitory binding of RuBP to allosteric sites on the enzyme. When added after RuBP, 6PG inhibits by competing for the catalytically active sites. This then suggests a complex regulatory mechanism for RuBP carboxylase. The insensitivity of non-high molecular enzymes may correlate with the absence of small subunits and also suggests a different catalytic site topography to that of the large carboxylases.

Other affectors of RuBP carboxylase have been reviewed by McFadden (1973). The influence of adenine nucleotides upon activity of the I. novellus enzyme (McCarthy and Charles, 1973) requires further investigation, particularly in view of their non-effect on the Ps. facilis RuBP carboxylase (McFadden and Tabita, 1974)

The presence of enzymes lacking small subunits indicates that the catalytic sites of RuBP carboxylase reside on the large subunits. This was also suggested by the finding of activity with the Chromatium D enzyme on removal of small subunits (Takabe and Akazawa, 1973). The isolated large subunits of the Chromatium enzyme also had RuBP-oxygenase activity. With spinach, antibody to the large subunit will inhibit both oxygenase and carboxylase activity whilst antibody to the small subunit will inhibit neither (Nishimura and Akazawa, 1974). What therefore, is the function of the small subunits?

Nishimura and Akazawa (1973) found that large subunit oligomers of spinach RuBP carboxylase, although able to function as a carboxylase, had an altered pH optimum which was no longer Mg^{2+} dependent. Antibodies to the small subunit also prevented Mg^{2+} dependent changes in pH optimum (Nishimura and Akazawa, 1974). A similar effect was reported by Takabe and Akazawa (1973) for the Chromatium D RuBP carboxylase.

The work of Chu and Bassham (1973) previously discussed, suggests the existence of regulatory sites on the enzyme and consequently, the role of the small subunits would seem to be in the regulation of enzyme activity. It has also been suggested that the small subunit is involved in the control of synthesis of the large subunit (Ellis, 1975). Further work is required to elucidate the nature of the small subunit mediated, regulation. A comparative examination of the regulatory properties of RuBP carboxylases with and without small subunits, may be worthwhile.

5. REGULATION OF THE CALVIN CYCLE

The regulation of the Calvin cycle would be expected to be of prime importance both in higher plants and algae during light dark transitions, involving the turning on and off of photosynthesis and in the many bacteria which exhibit heterotrophic and autotrophic metabolism. Regulation should also be evident due to the 'commonality' of many intermediate compounds of the cycle which may enter other metabolic pathways. Three enzymes function uniquely in the Calvin cycle; RuBP carboxylase, phosphoribulokinase and fructose/sedoheptulose bisphosphate phosphatase. The reactions these

enzymes catalyse are accompanied by large negative changes in free-energy, synonomous with sites of metabolic regulation (Atkinson, 1966), the role of these enzymes in the regulation of plant photosynthesis has been discussed by Walker (1976).

Inhibition by adenine monophosphate (AMP) of ribose 5-phosphate (R5P) : ATP dependent carbon dioxide fixation has been observed in cell-free extracts of Chromatium D (Johnson, 1966), R. spheroides (Rindt and Ohmann, 1969) and Thiobacillus thioparus (Johnson and Peck, 1965), this probably being due to inhibition of phosphoribulokinase. Reports have also indicated that β-NADH may be a positive effector of phosphoribulokinase in Nitrobacter winogradskyi (Kiewsow et al., 1977), R. spheroides (Rindt and Ohmann, 1969), Ps. facilis (MacElroy et al., 1969), R. rubrum (Joint et al., 1972) and Ps. oxalaticus (Knight et al., 1978). The degree of AMP inhibition in R. spheroides depends upon the concentration of β-NADH and Rindt and Ohmann (1969) have proposed that these effects provide a fine control by which the energy consuming Calvin cycle is only allowed to function if sufficient energy is available.

The importance of 6-phosphogluconate (6PG) as a modulator of RuBP carboxylase has already been indicated. 6PG is an important intermediate in both the oxidative pentose phosphate pathway and in the Entner-Doudoroff pathway.

The level of 6PG in the cell may reflect the function of these two pathways and so provide a control mechanism between oxidative heterotrophic metabolism and the reductive Calvin cycle.

There have been a number of studies into the control of synthesis of Calvin cycle enzymes particularly during transitions from heterotrophic to autotrophic metabolism. Many enzymes of the Calvin cycle are present in cell-free extracts at higher specific activity after autotrophic growth than after heterotrophic growth (Lascelles, 1960; Anderson and Fuller, 1967c; Tabita and McFadden, 1974a). Slater and Morris (1973a) have investigated how rates of carbon dioxide fixation and RuBP carboxylase activity are regulated in R. rubrum during autotrophic and heterotrophic growth in batch and continuous culture. They recognise three conditions: (i) during autotrophic growth high levels of RuBP carboxylase are paralleled by high rates of carbon dioxide fixation; (ii) during rapid growth in batch culture and in turbidostat continuous culture with malate as carbon source, a lower level of RuBP carboxylase is paralleled by a lower rate of carbon dioxide fixation; (iii) during malate limited growth in a chemostat relatively high rates of carbon dioxide fixation are accompanied by relatively low levels of RuBP carboxylase. They also found

that malate starvation of a heterotrophically grown culture, resulted in high rates of carbon dioxide fixation. They suggest that during heterotrophic growth, control of carbon dioxide fixation results from an internal level of malate or a metabolite derived from malate, effecting repression and inhibition of Calvin cycle enzymes. During chemostat growth the low level of growth limiting substrate allows relief of inhibition of carbon dioxide fixation but is not low enough to allow for complete drerepression of enzyme synthesis.

Ps. oxalaticus can grow on formate or oxalate as sole sources of carbon and energy, in both cases energy being derived from formate oxidation, oxalate being converted to formate through co-enzyme A thioesters. However, oxalate carbon is assimilated heterotrophically whilst formate carbon is assimilated autotrophically by a Calvin cycle (Quayle, 1961; Dijkhuizen et al., 1977). Blackmore and Quayle (1968) investigated how enzyme levels were affected by growth on mixed carbon substrates. In the presence of formate plus 'slow' growth substrates (those which supported a slower growth rate than formate), Calvin cycle enzymes were synthesised, this response suggesting that formate (or derivative) was an inducer of these enzymes. During growth on formate plus a 'fast' growth substrate (succinate, citrate, lactate), synthesis of Calvin cycle enzymes was repressed.

These results were confirmed and extended by the studies of Harder and his colleagues (Dijkhuizen *et al.*, 1978; Knight *et al.*, 1978) using substrate transition experiments and investigation of changes in pool sizes. Their work suggests that synthesis of Calvin cycle enzymes involves derepression/repression rather than an induction modulated by metabolite repression mechanism. This they suggest may be a common mechanism for the many bacteria able to use both autotrophic and heterotrophic modes of metabolism and is consistent with the results of Slater and Morris (1973a). Further evidence for depression/repression arises from the finding that RuBP carboxylase is synthesised in *Ps. oxalaticus* during oxalate limited growth in a chemostat at very low dilution rates (0.02 h^{-1} and less), in the absence of formate or other possible inducer (Knight *et al.*, 1978). Matin *et al.* (1976) have indicated the apparent release of metabolic repression at low growth rates and this may be a general phenomenon.

6. THE METABOLISM OF GLYCOLLATE

The production of 2-phosphoglycollate and thence glycollate in autotrophs as a result of RuBP oxygenase activity has already been indicated. Two other possible paths of glycollate synthesis have received attention in recent years: (i) the reductive condensation of two molecules of CO_2 and (ii) the oxidation of the two-carbon frgament of dihydroxyethylthiamine pyrophosphate, involved in the transketolase reaction of the Calvin cycle (Zelitch, 1975). No substantial role however, has been ascribed to either of these mechanisms.

Phosphoglycollate metabolism has been well studied in higher plants and algae although less work has been done with prokaryotic systems. Phosphoglycollate is dephosphorylated by a specific phosphatase (Anderson and Tolbert, 1966). Two major pathways of glycollate metabolism have been elucidated. In plants and green algae, glycollate is oxidised to glyoxylate and then metabolised via glycine, serine, hydroxypyruvate and glycerate in turn (Tolbert, 1971). A second route found in the cyanobacterium, Anabaena cylindrica (Codd and Stewart, 1973) involves metabolism via glyoxylate, tartronic semialdehyde and glycerate. This is also the pathway used by a Pseudomonas sp. during growth on glycollate (Kornberg and Gotto, 1961). Both of these pathways incur

loss of carbon dioxide. Pa. denitrificans grows on glycollate and glyoxylate by a β-hydroxyaspartate pathway involving condensation of glyoxylate and glycine to give β-hydroxy-aspartate avoiding this loss of carbon dioxide (Kornberg and Morris, 1965). In a fourth pathway, also found in Anab. cylindrica glyoxylate may be converted directly to malate by the action of malate synthase. These pathways are summarized in Figure 4.

The excretion of glycollate has been reported for a number of prokaryotes including R. rubrum, Anab. cylindrica (Codd and Smith, 1974) and Chromatium (Lorimer et al., 1978). Reasons for this excretion are unclear as is the answer to the question of whether excreted glycollate can be reabsorbed and used as a carbon source (Stanier and Cohen-Bazire, 1977).

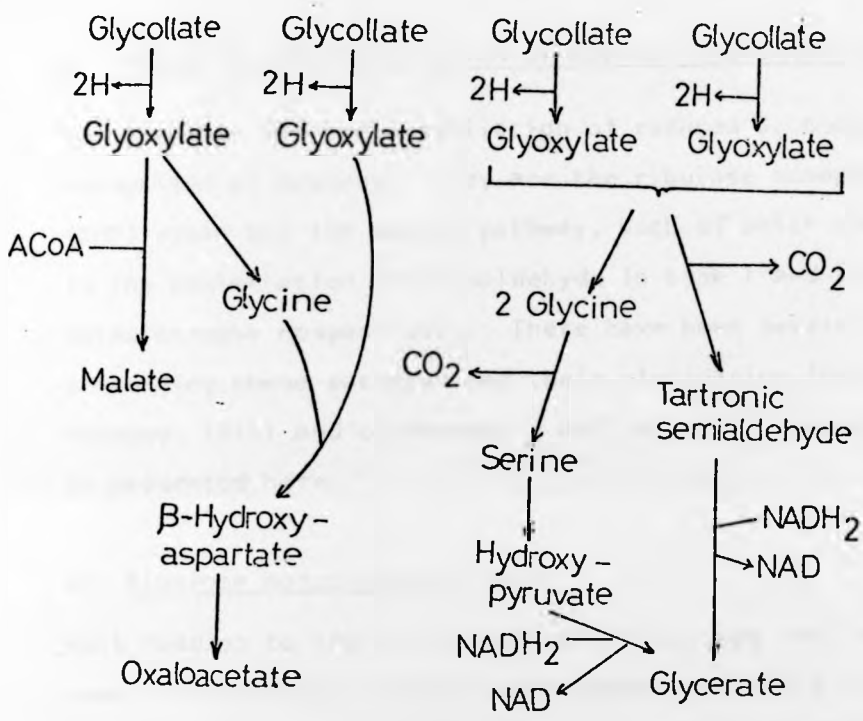


Fig. 4 Pathways of glycollate metabolism

7. PATHWAYS FOR ASSIMILATION OF REDUCED ONE-CARBON COMPOUNDS

Two pathways for the assimilation of reduced C_1 compounds are recognised at present. They are the ribulose monophosphate (RMP) cycle and the serine pathway, both of which are involved in the assimilation of formaldehyde in type I and type II methylotrophs respectively. There have been several reviews concerning these pathways and their elucidation (Quayle, 1972; Anthony, 1975) and consequently only a brief description will be presented here.

a) Ribulose monophosphate cycle

Work leading to the elucidation of this cycle was largely done with obligate methane and methanol utilising bacteria. In all variations of this cycle, two reactions remain unique; the condensation of formaldehyde with ribulose 5-phosphate (Ru5P), catalysed by hexulose phosphate synthase, and the isomerisation of the product of this reaction D-arabino-3-hexulose-6-phosphate, to yield fructose 6-phosphate catalysed by hexulose phosphate isomerase. The cleavage of fructose 6-phosphate to triose phosphate may occur either by FBP aldolase, as in glycolysis or by 2-keto-3-deoxy-6-phospho-gluconate (KDPG) aldolase as in the Entner-Doudoroff pathway (Fig. 5). Enzymes needed for cleavage by either routes are present in the methane oxidisers M. capsulatus and M. methanica

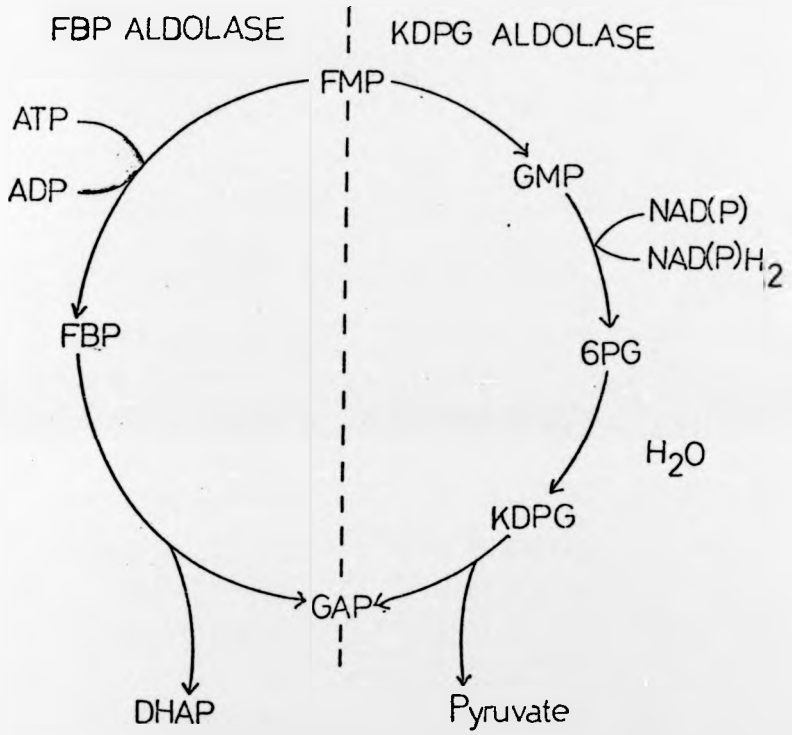


Fig. 5 Possible modes of cleavage of fructose 6-phosphate

Fig. 6 Ribulose monophosphate cycle
(after Quayle and Ferenci, 1978)

Abbreviations:
HuMP, D-arabino-3-hexulose-6-phosphate

Scheme involves SBP phosphatase but not transaldolase

FIXATION
HCHO
HuMP
FMP
ADP
ATP
DHAP
GAP
HCHO
HuMP
FMP
ADP
ATP
DHAP
GAP
HCHO
HuMP
FMP
CLEAVAGE
Ru5P
Ru5P
Ru5P
FMP
REARRANGEMENT
XuMP
EMP
SMP
P
SBP
R5P
XuMP

Fig. 7 Ribulose monophosphate cycle
(after Quayle and Ferenci, 1978)

Scheme involves transaldolase but not
SBP phosphatase.

(Strom et al., 1974). Variations also occur in the mode of regeneration of Ru5P depending upon the presence or absence of transaldolase and sedoheptulose 1,7-bisphosphate (SBP) phosphatase. The two overall schemes of the RMP cycle are shown in Figures 6 and 7. The methane oxidisers M. methanica and M. capsulatus, probably use the scheme shown in Figure 7 as these organisms contain transaldolase but not SBP phosphatase (Strom et al., 1974).

Mention should also be made of a dissimilatory cycle of formaldehyde oxidation which may be involved in the generation of reducing power. In addition to enzymes of the RMP cycle, 6PG dehydrogenase is required for this dissimilatory cycle, this enzyme being present in both M. methanica and M. capsulatus (Strom et al., 1974). Whether the cycle as shown in Figure 8, actually functions in vivo, is unclear.

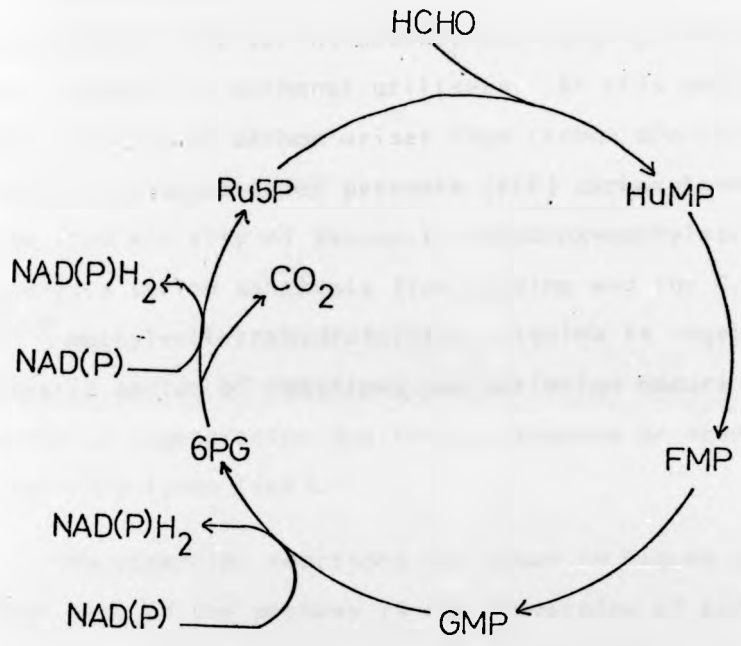


Fig. 8 Dissimilatory cycle of formaldehyde oxidation

b) Serine pathway

The nature of the serine pathway was largely worked out with facultative methanol utilisers. In this pathway, part of the cell carbon arises from carbon dioxide by activity of phosphoenol pyruvate (PEP) carboxylase and the rest from activity of serine transhydroxymethylase which catalyses serine synthesis from glycine and the C_1 carrier, $N^{5,10}$-methylenetetrahydrofolate. Glycine is regenerated by a cyclic series of reactions and variation occurs in the method of regeneration due to the presence or absence of isocitrate lyase (icl).

The essential reactions are shown in Figure 9. The final part of the pathway is the conversion of acetyl CoA to the C_3 and C_4 skeletons needed for biosynthesis of cellular material. Although these intermediates are available from the cycle itself, acetyl CoA needs to be converted to glyoxylate to replenish the cycle. Where isocitrate lyase is present (icl^+), acetyl CoA is further metabolised by glyoxylate cycle enzymes. However, because malate synthase would counteract the serine pathway enzyme, malyl CoA lyase, Harder et al. (1973) have suggested the pathway shown in Figure 10, for growth of Hyphomicrobium X on methanol.

Fig. 9 The serine pathway for methyoltrophic growth (after Anthony, 1975)

1. serine transhydroxymethylase
2. serine-glyoxylate aminotransferase
3. hydroxypyruvate reductase
4. glycerate kinase
5. enolase
6. phosphoenolpyruvate carboxylase
7. malate dehydrogenase
8. malate thiokinase
9. malyl CoA lyase

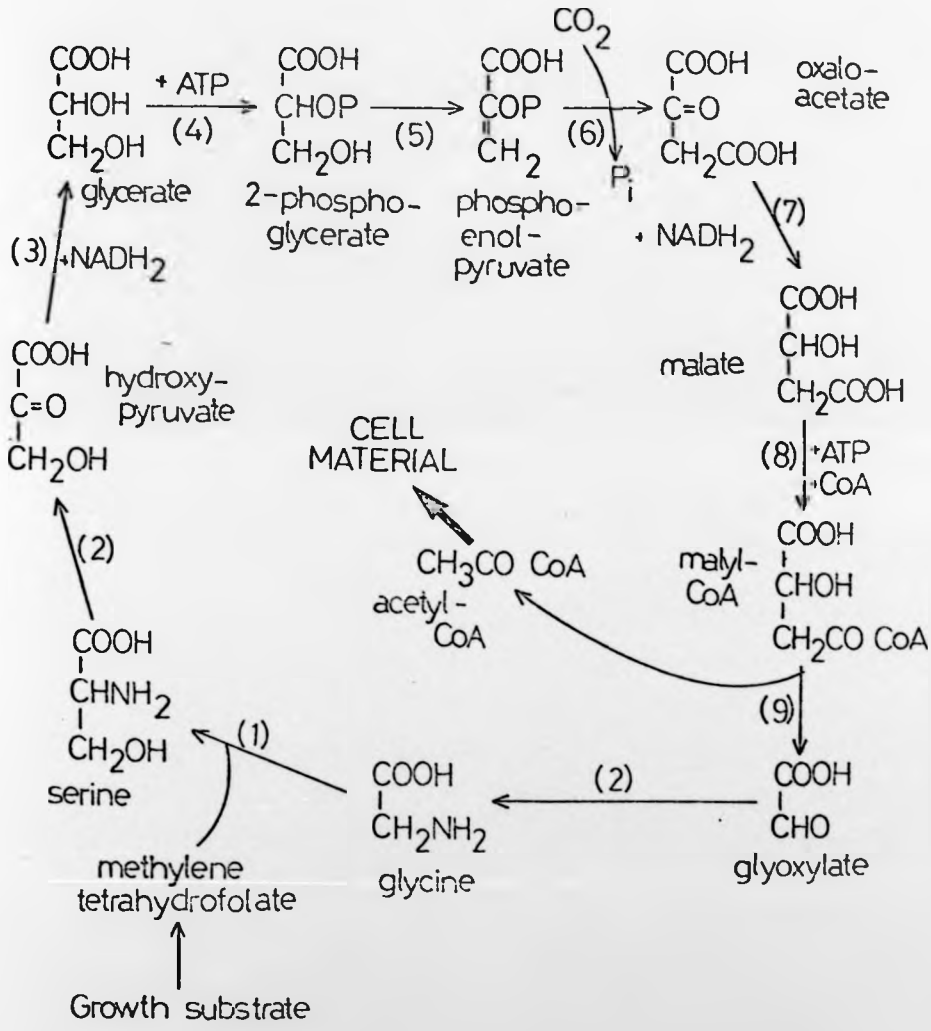
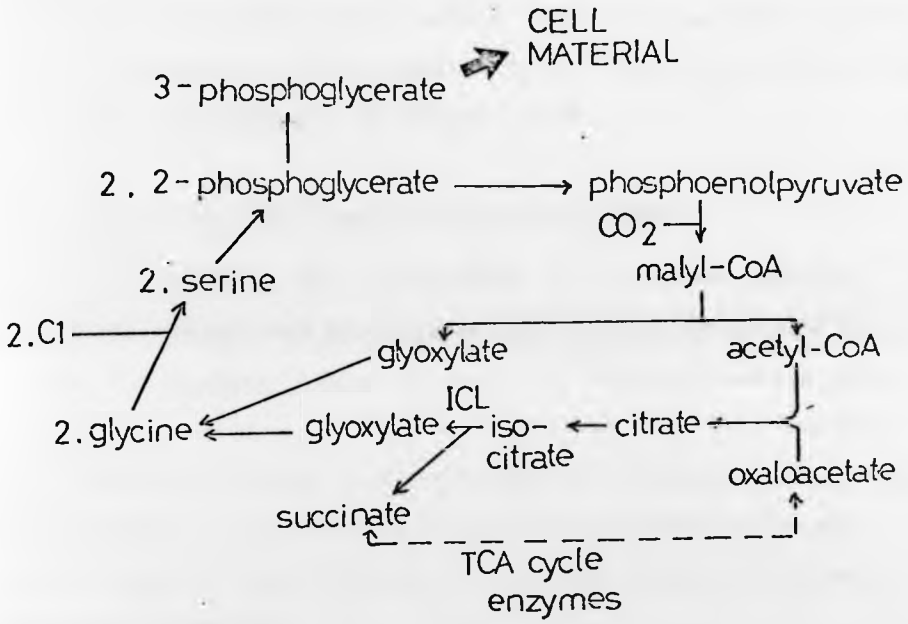

COOH
CHOH
CH2OH
+ ATP
(4)
COOH
CHOP
CH2OH
(5)
COOH
COP
CH2
CO2
(6)
Pi
COOH
C=O
CH2COOH
oxalo-
acetate
glycerate
2-phospho-
glycerate
phospho-
enol-
pyruvate
+ NADH2
(7)
(3)
+NADH2
COOH
CHOH
CH2COOH
malate
COOH
C=O
CH2OH
hydroxy-
pyruvate
CELL
MATERIAL
(8)
+ATP
+CoA
CH3CO CoA
acetyl-
CoA
COOH
CHOH
CH2CO CoA
malyl-
CoA
(2)
COOH
CHNH2
CH2OH
serine
(1)
(9)
COOH
CH2NH2
glycine
(2)
COOH
CHO
glyoxylate
methylene
tetrahydrofolate
Growth substrate

Fig. 10 Scheme for 'C_1' assimilation in Hyphomicrobium growing on methanol (after Harder et al., 1973)



Abbreviations : ICL - isocitrate lyase

Where isocitrate lyase is absent (icl^-), such as in Pseudomonas AM1, the situation is less well understood and the method of conversion of acetyl CoA to glyoxylate unknown. However, malate synthase appears to be involved and glycollate may be an intermediate (Anthony, 1975).

c) Carbon dioxide fixation by methylotrophs

The involvement of PEP carboxylase in the serine pathway was first recognised by Large et al. (1961) working with Ps AM1 and Hyphomicrobium vulgare. [^{14}C]-bicarbonate was initially incorporated into C_4 acids and glycine. However, [^{14}C]-formate (which is assimilated as CO_2 by many organisms) was initially incorporated into phosphorylated compounds which suggests that this compound is not totally oxidised to carbon dioxide.

In M. methanica, which has an RMP cycle for carbon assimilation, Johnson and Quayle (1965) found little assimilation of [^{14}C]-bicarbonate, with aspartate and malate being the major, early labelled intermediates. Very little incorporation was also reported from [^{14}C]-formate (Kemp and Quayle, 1967).

The incorporation of [^{14}C]-bicarbonate and [^{14}C]-formate by M. capsulatus has been examined by Eccleston and Kelly (1973). The quantitative importance of these substrates as sources of carbon was not assessed but after one hours incubation, labelling of aspartate was taken to suggest carboxylation of C_3-substrates. The short term incorporation of [^{14}C]-formate by M. capsulatus was studied by Reed (1976) and although only small quantities of this substrate were assimilated, the early labelling of serine and glycine was considered to indicate direct incorporation without prior oxidation to carbon dioxide.

In summary, carbon dioxide assimilated by methylotrophs generally involves carboxylation of C_3 substrates. There is no published report implicating RuBP carboxylase or a Calvin cycle in carbon dioxide fixation by a methylotroph.

8. ENERGETICS OF C_1 ASSIMILATION PATHWAYS

The three pathways of C_1 assimilation described above have very different energy requirements and these may be best compared by normalizing all three to pyruvate production using established glycolytic steps to convert triose phosphate and 3-phosphoglycerate to pyruvate (Table 2).

Of the four possible variations of the RMP cycle, only the eda^+/sda^+ variant has not been found to date and this correlates with its relatively high energy requirement. Although there are two possible rearrangement sequences in the Calvin cycle, only the tal^-/sda^+ variant has been found to operate. Only the (icl^+)-serine pathway is considered in view of the doubt regarding the (icl^-)-serine pathway.

The far greater amount of energy needed to assimilate carbon dioxide rather than formaldehyde is very apparent from Table 2 as is the greater energy cost of assimilating formaldehyde by a serine pathway rather than by an RMP cycle.

The potential cell yields of organisms growing on C_1-compounds have been calculated by a number of workers (van Dijken and Harder, 1975; Anthony, 1978). For a given P/O ratio (mol ATP formed per electron pair transferred to oxygen), precise values for which are unclear, all predict that growth yields following carbon assimilation via the serine pathway would be lower than when using the RMP cycle.

Table 2 Energy requirements for C_1-assimilation pathways normalized to pyruvate production (after Quayle and Ferenci, 1978)

Cycle	Cleavage		Rearrangement		Reactants	Product	$DNAD(P)H_2$	$DFPH_2$	DATP
	FDP aldolase (fda)	KDPG aldolase (eda)	trans-aldolase (tal)	SDPase (sda)					
RMP	-	+	-	+	3HCHO		+1	0	-3
	-	+	+	-	3HCHO		+1	0	0
	+	-	-	+	3HCHO		+1	0	0
	+	-	+	-	3HCHO		+1	0	+1
icl^+-serine					2HCHO + $1CO_2$	PYRUVATE	-2	+1	-2
Calvin			-	+	$3CO_2$		-5	0	-7
			+	-	$3CO_2$		-5	0	-7

Abbreviations: (fda), fructose bisphosphate aldolase; (eda), 2-keto-3-deoxy-6-phosphogluconate aldolase; (tal), transaldolase; (sda), sedoheptulose bisphosphate phosphatase; FP, flavoprotein of succinate dehydrogenase.

Furthermore, Anthony (1978) indicated that P. denitrificans growing on methanol by a Calvin cycle, only shows approximately half of the cell yield it would have if methanol was assimilated by an RMP cycle.

Anthony (1978) also introduced the concept of NAD(P)H limitation of growth yields. Most heterotrophs require little NAD(P)H for conversion of growth substrate to precursors for biosynthesis and their molar growth yields are predominantly determined by the ATP supply. When however, there is a high NAD(P)H requirement for assimilation of growth substrate (as with CO_2 and other C_1-compounds), then growth will be either totally NAD(P)H limited or both NAD(P)H and ATP limited. This former condition becomes effective in many methylotrophs where formate dehydrogenase may be the only NAD(P)H linked, oxidative enzyme. In these organisms, the dissimilatory cycle of formaldehyde oxidation (Fig. 8) may have particular importance as a source of NAD(P)H.

9. AUTOTROPHY AND ITS EVOLUTION

A number of articles have been published in recent years speculating as to the possible line of evolution of C_1-metabolism (Kelly, 1971; McFadden, 1973; McFadden and Tabita, 1974; McFadden, 1975; Quayle and Ferenci, 1978). Given that the earliest self-reproducing organisms utilised ready made materials, it is of interest to determine how autotrophy, and particularly the Calvin cycle, developed from these early heterotrophs.

Reference to Table 2 indicates the endergonic nature of cellular biosynthesis from carbon dioxide in contrast to biosynthesis from the reduced C_1-compounds which may be exergonic. Therefore growth on carbon dioxide requires enzymes of carbon assimilation and a system of energy generation. It then seems likely that utilisation of reduced C_1-compounds is more primitive than utilisation of carbon dioxide and this assumption has been used by most authors.

Quayle and Ferenci (1978) have suggested that the RMP cycle was the basis for the phase of evolution following the early heterotrophs. Several features of the RMP cycle commend it as a primitive pathway, notably the ability to assemble it to give a simple fermentation of formaldehyde (10), a compound readily formed in experiments simulating the prebiotic

environment. A fermentation mechanism is essential as molecular oxygen is not believed to have been present until relatively late in evolution, arising as a result of photosynthesis.

(10) $3HCHO + ADP + Pi \longrightarrow lactate + ATP$

The development of the Calvin cycle essentially reduces to a consideration of the two enzymes unique to the cycle, RuBP carboxylase and phosphoribulokinase. Other enzymes of the cycle have analogous functions in glycolysis and the pentose phosphate pathways which, because they occur in most anaerobes, were probably established before the acquisition of these two enzymes. McFadden (1973) has proposed that phosphoribulokinase activity arose from phosphofructokinase. However, no studies have been published to support this idea.

McFadden (1975) has discussed the clear similarity between RMP and Calvin cycles and indicated the homology between hexulose phosphate synthase and RuBP carboxylase both of which catalyse condensation of C_1-compounds with C_5-ketose phosphates. However, mechanistically these enzymes are quite different catalysing an aldol condensation and carboxylation of an enolate respectively. Quayle and Ferenci (1978) indicating this fact, suggested that carboxylation of phosphoenolpyruvate (PEP) was a more likely precursor of RuBP

carboxylase and also that a primative RuBP carboxylase may have used ribulose 5-phosphate (Ru5P) as substrate giving glycerate and phosphoglycerate as products. The apparent unimportance of the C_1-phosphate group on RuBP supports this idea. Further light may be shed on this subject with a study of substrate specificity and inhibition studies of PEP and RuBP carboxylases.

Most evolutionary arrangements of autotrophs suggest that the Calvin cycle was initially present in anaerobes utilising light or inorganic redox reactions as energy sources. The biological photolysis of water presumably then followed as in the cyanobacteria, green algae and higher plants. This pattern of development is paralleled by increasing molecular weight of RuBP carboxylase (Table 1). Furthermore, because of the structural homology of the large subunit in contrast to the diverse structures of the small subunit, the suggestion that these have arisen at different times in evolution has received much attention and has led to postulated schemes of evolution (Fig. 11; McFadden, 1975) with the genes for the large subunit being first established.

In developing schemes for the evolution of the Calvin cycle, the similarity between this and the RMP cycle becomes very apparent. Many other properties of methane oxidisers indicate a relationship to those organisms with a Calvin cycle

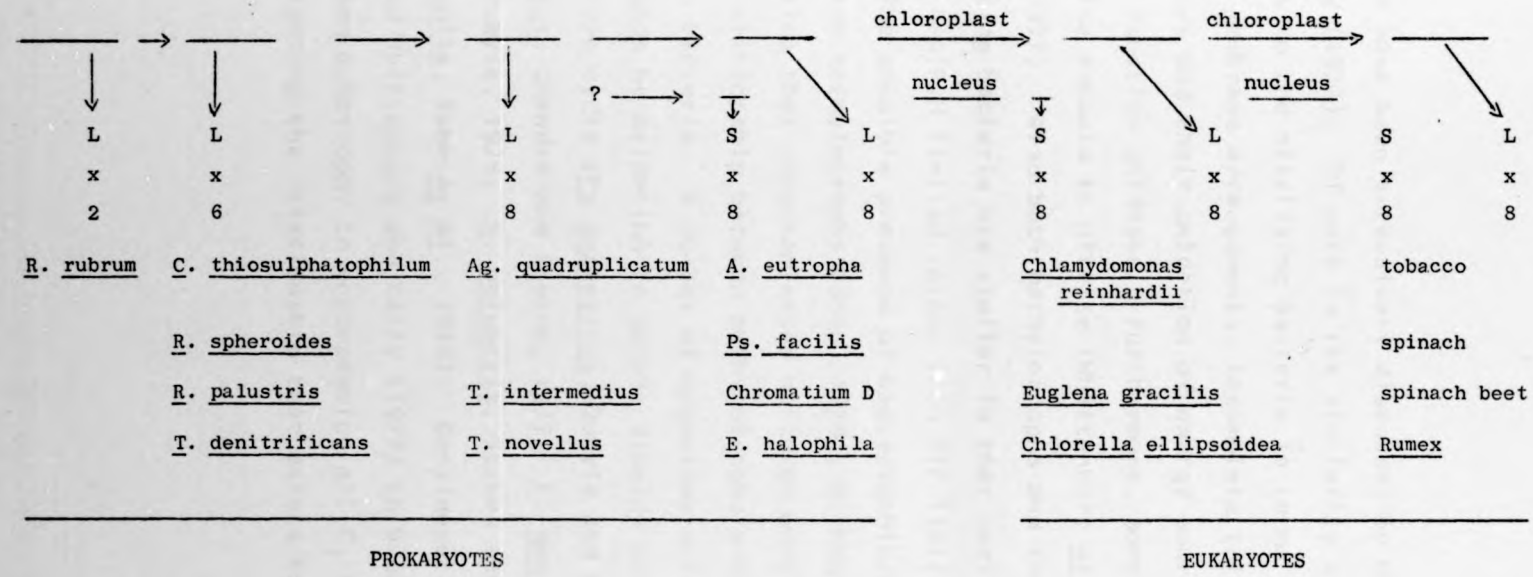


Fig. 11 Evolutionary scheme for structural gene(s) specifying RuBP carboxylase (after McFadden, 1975)

L, large subunit; S, small subunit

and these have been extensively discussed by Whittenbury and Kelly (1977). Of note is the similarity of the methane oxidisers to the nitrifying bacteria in terms of their complex internal membrane arrangements, incomplete tricarboxylic acid cycles and their oxidation of energy sources (CH_4, NH_3) by mixed function oxidases. Furthermore, some methylotrophs can oxidise ammonia to nitrite (Whittenbury et al., 1970; Dalton, 1977). Also both methylotrophs and the carbon dioxide assimilating bacteria are similar in that their growth is probably NAD(P)H limited rather than ATP limited (Anthony, 1978). The possible presence of bacteriochlorophyll in two facultative methylotrophs (Sato, 1978), although needing confirmation that uncontaminated cultures were used, suggests a close relationship between methylotrophs and the photosynthetic bacteria. A number of organisms utilise reduced C_1 compounds by oxidation to carbon dioxide before assimilation via a Calvin cycle (Ps oxalaticus, Quayle and Keech, 1959; T. novellus, Chandra and Shetna, 1977; P. denitrificans, Cox and Quayle, 1975; R. palustris, Stokes and Hoare, 1969; R. acidophila, Sahm et al., 1976). Consideration of these points led Whittenbury and Kelly (1977) to broaden the scope of the term autotrophy to encompassing all C_1-utilisers, and so highlighting the relationship that exists between them.

SECTION II

MATERIALS AND METHODS

1. ORGANISMS

Rhodomicrobium vannielii strain (RM5), isolated by Dr. C. S. Dow (University of Warwick) and Rhodospirillum rubrum (University of Warwick culture collection) were the species of Rhodospirillaceae used in this study. Methylococcus capsulatus strain (Bath) was isolated by Whittenbury et al. (1970). Other methylotrophs used were: Methylococcus capsulatus (Foster and Davis); Methylomonas albus (BG8); Methylosinus trichosporium (OB3b); Methylosinus sporium (5); Methylomonas methanica (S1); Methylomonas agile (A30) and Methylocystis parvum (CBBP) all from the University of Warwick culture collection. The trimethylamine utilisers, bacterium C2A1 and bacterium 4B6 were isolated by Colby and Zatman (1973).

2. CHEMICALS AND GASES

RuBP tetrasodium salt, other biochemicals and enzymes were purchased from the Sigma London Chemical Co. Ltd., Poole, Dorset, U.K., except for reduced nicatinomide adenine dinucleotide (NADH) which came from the Boehringer Corporation (London) Ltd. Fluka AG, Switzerland, supplied acrylamide and the Eastman Kodak Co., Kirkby, Liverpool, U.K., N,N'-Methylene bisacrylamide and N,N,N',N'-tetramethyl ethylenediamine (TEMED).

Chemicals for electron microscopy were obtained from Polaron Equipment Ltd., Watford, U.K., except for lead citrate which came from TAAB Laboratories, Reading, U.K. All other chemicals were obtained from the following manufacturers: British Drug Houses Ltd., Poole, Dorset, U.K., Fisons Scientific Apparatus Ltd., Loughborough, Leics, U.K., Hopkin and Williams, Chadwell Heath, Essex, U.K., G. T. Gurr Ltd., High Wycombe, Bucks, U.K. Radiochemicals were purchased from the Radiochemical Centre, Amersham, U.K.

Oxygen free nitrogen, air, methane, carbon dioxide and helium were obtained from the British Oxygen Co. Ltd., London, U.K.

3. MEDIA

R. vannielii (RM5) was routinely grown on a medium of the following composition ($g.l^{-1}$):

PM Medium (Dow, 1974)

NaCl	0.4
NH_4Cl	0.5
$CaCl_2.2H_2O$	0.05
$MgSO_4.7H_2O$	0.4
sodium hydrogen malate	1.5
sodium pyruvate	1.0

adjusted to pH 6.8 with KOH

After autoclaving at 121°C for 15 min, 50 $ml.l^{-1}$ of presterilised phosphate buffer (0.1M, pH 6.8) was aseptically added. Media containing other carbon substrates were prepared using the same basal salts, omitting sodium hydrogen malate and sodium pyruvate and including the required substrate at a final concentration of 20 mM. When necessary, carbon dioxide was provided by the addition of filter sterilised sodium bicarbonate. For growth of R. rubrum PM medium was used but in addition containing 1 $g.l^{-1}$ of yeast extract.

For methylotrophs, a medium of the following basal salts composition was used ($g.l^{-1}$):

$MgSO_4.7H_2O$	1.0
$CaCl_2.2H_2O$	0.2
ethylenediamine tetracetic acid ferric sodium salt	0.005

To this was added 0.5 $ml.l^{-1}$ of a trace element solution (Dalton and Whittenbury, 1976). Nitrate mineral salts medium (NMS) contained in addition 1 $g.l^{-1}$ of KNO_3 and ammonium mineral salts medium (AMS), between 0.25 to 2.0 $g.l^{-1}$ NH_4Cl. After adjustment to pH 6.8 with KOH and autoclaving at 121°C for 15 min, 20 $ml.l^{-1}$ of presterilised phosphate buffer (0.2M, pH 6.8) was added.

For solid media, 15 $g.l^{-1}$ of 'Bacto Difco' agar was added prior to sterilisation of the unbuffered medium.

4. MAINTENANCE OF CULTURES

R. vannielii (RM5) and M. capsulatus (Bath) were both regularly subcultured on PM agar and NMS agar respectively. R. vannielii (RM5) was grown in anaerobic bags (Westmacott and Primrose, 1975) at 30° under illumination from tungsten lamps. Incubation of M. capsulatus plates was at 45°C in

'Tupperware' boxes, fitted with air tight lids, into each of which a football bladder of methane was discharged. This gave an approximate 50 : 50 methane : air mixture. Cultures of other organisms were stored at -80°C.

5. CULTURE PURITY

The purity of all cultures was checked by streaking on nutrient agar plates and incubating these both aerobically and anaerobically at 30°C and 45°C. Cultures were also examined by phase contrast microscopy.

6. MICROSCOPY

a) Light microscopy

Phase contrast microscopy was done using an Olympus EMT microscope. Plates were examined with an Olympus 'Model X-Tr' stereoscopic microscope.

b) Transmission electron microscopy

This was done with an AEI Corinth 275 Electron Microscope having an accelerating voltage of 60KV. Electron micrographs

were taken on 70 mm Ilford line film N4E50, developed in Ilford Phenisol and fixed in Kodafix. Prints were made using Kodak Bromide paper.

c) Negative staining

Cells or enzyme preparations were placed on formvar coated grids, fixed, if required, in osmium tetroxide vapour and dried down. 1% (w/v) phosphotungstic acid (pH 7.0) was added and immediately removed with filter paper.

7. SPECTROPHOTOMETRY

All spectrophotometric enzyme assays were done using a Pye-Unicam SP1800 recording spectrophotometer fitted with a constant temperature cuvette housing and linked to a Unicam AR25 linear recorder. 10 mm light path, glass or quartz cuvettes were used. Measurement of culture absorbances at 540nm (A_{540}) and Lowry *et al*. (1951) protein determinations, were done on a Pye-Unicam SP500.

8. PROTEIN DETERMINATIONS

Protein concentration in cell-free extracts was determined by the Method of Lowry *et al*. (1951) as modified by Kennedy and Fewson (1968). Dried, crystalline bovine plasma albumin was used as standard. Absorbance of solutions at 280 nm (A_{280}) was used as a qualitative estimation of protein concentration.

9. DRY WEIGHT ESTIMATIONS

Measurement of culture dry weights was done by filtration of suitable volumes through dried and weighed 0.4 μ membrane filters (Oxoid Ltd., London). These were then dried at 60°C under identical conditions to the fresh membranes and allowed to cool in a desiccator before weighing. On average 20-30 mg dry weight of cells were filtered.

By dilution of chemostat grown cells, curves of A_{540} *versus* dry weight were prepared for both *M. capsulatus* (Bath) and *R. vannielii* (RM5). These gave average dry weight values at an A_{540} of 0.1 of 0.016 $g.l^{-1}$ for *M. capsulatus* (Bath) and 0.023 $g.l^{-1}$ for *R. vannielii* (RM5).

10. GAS CHROMATOGRAPHY PROCEDURES

Measurement of carbon dioxide, methane, oxygen and nitrogen in culture atmospheres was done using a Pye-Unicam Series 104 gas chromatograph fitted with a Katharometer (thermal conductivity) detector. This was linked to a 'Servoscribe' recorder (Smiths Industries Ltd., London) with an integration device for calculating peak areas. 3 m x 4 mm columns were used with a packing material of either 'Molecular Sieve' 5A (80-100 mesh) for resolving oxygen, nitrogen and methane or 'Porapak R' for resolving air, methane and carbon dioxide. In both cases helium was used as the carrier gas at a flow rate of 30 $ml.min^{-1}$ and the oven temperature was maintained at $50^{o}C$.

Concentrations were expressed as a percentage of the total gas atmosphere. Due to differences in detector sensitivity towards various gases, it was necessary to calibrate the system with prepared gas mixtures of known composition.

11. MEASUREMENT OF RADIOACTIVITY

Radioactivity was counted on a Packard Tri-Carb Liquid Scintillation Spectrometer model 3320. The scintillation fluid used throughout was of the following composition:

6 l. toluene AR; 3 l. triton X-100; 36 g 2,5-Diphenyl-oxazole (PPO); 0.45 g p-Bis 2-(5-phenyloxazolyl)-benzene (POPOP). 10 ml of this scintillation fluid was completely miscible with 1 ml of water. A counting efficiency of 60% was calculated using ^{14}C standards of known specific activity.

12. PREPARATION OF RADIOACTIVE MATERIALS

Before use [^{14}C]-sodium bicarbonate was made up to a specific activity of 0.8 μCi μmoi^{-1} in 20 mM Tris (Hydroxymethyl)amino-methane hydrochloride (Tris-HCl), pH 8.2. [^{14}C]-formaldehyde was made up to a specific activity of 0.01 μCi μmol^{-1} in 10 mM Tris-HCl, pH 7.2.

Sodium [I-^{14}C]glycollate was dissolved in the minimum quantity of water and purified by paper chromatography on Whatman No. 1 paper in an ethanol-ammonia-water (80 : 4 : 16; Long et al., 1951) solvent system.

13. POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

a) Sodium dodecyl sulphate (SDS) - PAGE

The procedure of O'Farrell (1975) was followed for the preparation and running of 20 x 18 x 0.15 cm slab SDS-polyacrylamide gels. A discontinuous buffer system (Laemmli, 1970) was used with the resolving gel prepared in 1.5 M Tris-HCl, pH 8.8 and the stacking gel in 0.5 M Tris-HCl, pH 6.8. SDS at 0.4% (w/v) was incorporated into both buffers. The running buffer was Tris-glycine (0.025M Tris base, 0.192M glycine) containing 0.1% (w/v) SDS and 10 mM 2-mercaptoethanol. Either 7.5 to 20% (w/v) exponential gradient acrylamide gels or, for measuring polypeptide molecular weights, single percentage, 10% (w/v) acrylamide gels were used. Electrophoresis was done at a constant current of 12 ma.

b) Non-denaturing gels

Enzyme purity was assessed with non-denaturing tube gels (Gabriel, 1971). The Laemmli (1970) discontinuous buffer system was used with the addition of cysteine-HCl (final concentration, 8 mM) and 2-mercaptoethanol (final concentration, 10 mM) to the running buffer. Gels were polymerised from 4, 4.5, 5, 6 and 7.5% (w/v) acrylamide with a 1.5% (w/v) acrylamide stacking gel when required. Electrophoresis was done at 0.5 ma per tube and then increased to 2.5 ma per tube when the proteins had entered the gel.

c) Sample preparation

Soluble proteins for SDS-PAGE were dissociated in 1% (w/v) SDS, 0.1% (w/v) 2-mercaptoethanol and 6% (w/v) sucrose, by heating at 100°C for 3 mins. Soluble proteins for non-denaturing gels were prepared in 6% (w/v) sucrose plus 0.01% (w/v) 2-mercaptoethanol. Prior to loading onto gels, a tracking dye, bromophenol blue (BPB), was added to each sample (20 µl 0.1% w/v BPB/0.5 ml sample).

d) Gel staining and recording

Gels were stained for a minimum of 12 h in 0.1% (w/v) Coomassie Blue R, 45% (v/v) methanol and 10% (v/v) glacial acetic acid. Destaining was done in 45% (v/v) methanol, 10% (v/v) acetic acid followed by 20% (v/v) isopropanol, 10% (v/v) acetic acid and finally in 10% (v/v) isopropanol, 10% (v/v) acetic acid. Gels were photographed from above using a Pentax SP500 camera with Kodak Panatomic X film (ASA 32).

e) Standards

The marker proteins used for SDS-PAGE were: bovine plasma albumin (mol. wt. 67,000), γ-globulins (mol.wts. 25,000 and 50,000), ovalbumin (mol. wt. 43,500), horse heart myoglobin (mol. wt. 16,890) and horse heart cytochrome C (mol. wt. 13,400). Relative fronts (R_F) were measured with respect to bromophenol blue.

14. GROWTH OF ORGANISMS

a) Batch Culture

Photosynthetic bacteria were routinely grown in 250 ml Erlenmeyer flasks fitted with B19 ground glass sockets and sealed with rubber serum caps (W. Freeman and Co. Ltd., Barnsley, Yorks). The culture volume was 100 ml unless indicated otherwise. Each flask was gassed for 15 min with oxygen free nitrogen via 'inlet' and 'outlet' syringe needles inserted through the cap and then incubated on an orbital shaker (L. H. Engineering, Stoke Poges, Bucks) at 30°C under illumination from tungsten lamps (incident light intensity 2000 lux). The effect of growth substrate on enzyme activity was determined using cultures of 200 ml final volume prepared as above. Inocula were taken from cultures grown on each type of medium, the cells being washed once with 20 mM phosphate buffer, pH 7.0 prior to inoculation to an A_{540} of 0.1.

For enzyme studies and following growth curves, *R. vannielii* (RM5) and *R. rubrum* were grown in 5 l. or 20 l. flasks, sealed with rubber serum caps and flushed with oxygen free nitrogen. These were incubated with stirring by a magnetic follower at 30°C under constant illumination from tungsten lamps (incident light intensity of 2000 lux).

Small scale batch cultures of methylotrophs were grown in 250 ml flasks sealed with rubber serum caps and containing 20 ml of NMS medium and 25 ml of methane. Cell-free extracts of different methylotrophs were prepared from cells grown in 1 l. sealed flasks, each containing 400 ml of NMS medium, with a methane filled football bladder attached. Cultures were incubated with shaking at either 45°C for M. capsulatus (Bath) or 30°C for other methylotrophs.

Large scale batch culture of M. capsulatus (Bath) for enzyme studies, was done by P. P. Taylor (University of Warwick) in a 100 l. fermenter (L. H. Engineering Ltd.) as described by Colby et al. (1977).

b) Continuous Culture

The continuous culture of R. vannielii (RM5) was based on the techniques of Baker (1968) and France (1978) with a 1 l. 'Quickfit' fermenter vessel having a working volume of 600 ml. The culture was maintained at 30°C by a water jacket (5 l. glass beaker) connected to a thermocirculator (Churchill Instrument Co. Ltd., Perivale, Middlesex) and constant light intensity provided by a tungsten lamp. pH was maintained at 7.0 by the automatic addition of carbon dioxide. The culture was continuously flushed with oxygen free nitrogen (30 ml min^{-1}) and a gas lift was used to keep a constant culture volume. Agitation was provided by a magnetic follower.

For all radiotracer work and some enzyme studies, M. capsulatus (Bath) was grown as a chemostat culture (maintained by P. P. Taylor, University of Warwick) on AMS-medium (unless indicated otherwise) in a 3 l. fermenter (L. H. Engineering Ltd.) with a working volume of 2-2½ l. This was operated at 45°C under oxygen limitation with methane (20% v/v in air) as carbon source. The pH maintained at 6.8 by automatic titrations with 1M HCl. A dilution rate of 0.05 h^{-1} was used in all experiments.

15. MEASUREMENT OF CARBON DIOXIDE ASSIMILATION BY INTACT ORGANISMS

The procedure used for measuring whole cell carbon dioxide uptake by R. vannielii (RM5) was based on that of Slater and Morris (1973a). Cells were harvested by centrifugation (10,000 g for 10 min), washed once and resuspended to a standard A_{540} of 0.1 in either complete growth medium or basal salts, at 30°C. The suspension (20 ml in a 250 ml Erlenmeyer flask sealed with a rubber serum cap) was gassed for 10 min with oxygen free nitrogen and then equilibrated at 30°C for 10 min in a shaking water bath (Grant Instruments Ltd., Cambridge, U.K.) at 80 cycles.min^{-1} under illumination from tungsten lamps (incident light intensity, 2000 lux). For dark incubation, flasks were wrapped in aluminium foil. Sodium

bicarbonate, at a final concentration of 2.5 mM, was then added and after a further 10 min, 10 μCi of [^{14}C]-sodium bicarbonate to give a specific activity of 0.2 μCi.μmol^{-1}. At regular time intervals (10 min), a 1.0 ml sample was transferred to a scintillation vial containing 2.0 ml of 95% (v/v) ethanol, 5% (v/v) glacial acetic acid. Samples were evaporated to dryness on a Techne Dri Block DB-3H, resuspended in 1.0 ml of distilled water and radioactivity (fixed ^{14}C) measured.

A similar procedure was adopted for measuring carbon dioxide uptake by M. capsulatus (Bath) except that the suspension was not gassed with oxygen free nitrogen and 25ml of methane was added to each flask. Incubation was at 45^{o}C and illumination was not required.

Control flasks either contained heat killed cells (80^{o}C for 5 min) or lacked any exogeneous carbon source. Rates of carbon dioxide fixation were determined from the linear incorporation over 10-60 min and were expressed as μmol carbon dioxide fixed.mg dry wt. of organism^{-1}.h^{-1}.

16. PREPARATION OF EXTRACTS

All operations were done at 4°C. Cells were harvested by centrifugation at 10,000 g, washed twice and resuspended in 20 mM Tris-HCl, pH 8.0 containing 10 mM $MgCl_2.6H_2O$, 50 mM $NaHCO_3$, 1 mM ethylene diamine tetra-acetic acid, di-sodium salt (EDTA) and 5 mM 2-mercaptoethanol (TEMMB buffer; Tabita and McFadden, 1974a). Crude cell extracts were prepared by two passages of the suspension through a pre-cooled French Pressure Cell (American Instrument Co., Maryland, U.S.A.) at 137 MPa (20,000 lb in^{-2}). Unbroken cells and debris were removed by centrifugation at 10,000 g for 10 min and the supernatant was centrifuged at 120,000 g for 1½ h to yield soluble and particulate fractions.

Extracts of M. capsulatus (Bath) grown as a large scale batch culture (100 l. fermenter), were made using the method of Colby and Dalton (1976). Cells were harvested, washed twice and resuspended in 20 mM Tris-HCl buffer, pH 7.0. The suspension was passed once through a French Pressure Cell at 137 MPa and unbroken bacteria and debris removed by centrifugation at 5000 g for 10 min. The crude extract was then centrifuged at 160,000 g for 1 h to yield a dark red particulate fraction (P_{160}) and a clear red supernatant (S_{160}).

Locally bought spinach was homogenised in a Waring Blender for 2 min (100 g spinach plus 50 ml TEMMB buffer) and then centrifuged (100,000 g for 1½ h) to yield a clear supernatant.

17. MEASUREMENT OF CELL-FREE CARBON DIOXIDE FIXATION

Carbon dioxide fixation by cell-free extracts was measured in a reaction mixture (0.25 ml final volume) which contained: 15 μmol Tris-HCl, pH 8.2; 2.5 μmol $MgCl_2.6H_2O$; 5 μmol $NaH^{14}CO_3$ (specific activity 0.8 μCi μmol^{-1} unless stated otherwise); soluble extract; when required NADH and adenine triphosphate (ATP) (0.2 μmol of each). After preincubation for 5 min at the desired temperature, the reaction was started by the addition of 0.2 μmol of the test substrate. The reaction was stopped after a further 5 min by the addition of 100 μl of 12M formic acid and any precipitate removed by centrifugation. A 200 μl sample was evaporated to dryness in a scintillation vial, resuspended in 1 ml of water and radioactivity counted. Controls assayed simultaneously lacked any test substrate.

18. ENZYME ASSAYS

All enzyme assays were done at 45°C for *M. capsulatus* (Bath) and 30°C for other methylotrophs, *R. vannielii* (RM5) and *R. rubrum*.

a) *RuBP carboxylase* (3-Phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39): activity was measured by the RuBP dependent incorporation of [^{14}C]-bicarbonate into acid

stable products using the procedure described above for cell-free carbon dioxide fixation. A pH of 8.2 was used for R. vannielii (RM5), 7.8 for R. rubrum and 7.6 for both Methylococcus species. Any inhibitors, etc. were added to the reaction mixture prior to initiation of the reaction with RuBP

As an alternative, assays were done in 5 ml conical flasks containing 1 ml of standard reaction mixture. If required, these were made anaerobic by flushing with oxygen free nitrogen and sealing with rubber serum caps. After initiation of the reaction, 100 µl samples were transferred at intervals to scintillation vials containing 100 µl 12M formic acid. Radioactivity was then determined as before.

b) RuBP oxygenase: this was assayed by measuring the initial rate of oxygen uptake with a Clark-type oxygen electrode (Rank Bros., Cambridge). The reaction mixture contained (1.0 ml final volume), 60 µmol Tris-HCl, pH 7.6; 10 µmol $MgCl_2.6H_2O$; enzyme solution. After 5 min pre-incubation at the desired temperature, the reaction was started by the addition of 0.8 µmol RuBP.

c) Phosphoribulokinase (ATP: D-ribulose-5-phosphate 1-phosphotransferase, EC 2.7.1.19): activity was measured using ribose-5-phosphate (R5P) or ribulose-5-phosphate (Ru5P) as test substrate for the cell-free carbon dioxide fixation

assay described above. ATP (0.2 μmol) and when required, NADH (0.2 μmol) and purified R. vannielii (RM5) RuBP carboxylase, were included in the reaction mixture.

d) Phosphoenolpyruvate (PEP) carboxykinase (GTP: oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32): this was assayed by measuring the rate of incorporation of [^{14}C]-bicarbonate into malate in the presence of NADH and malate dehydrogenase (Sahl and Truper, 1977).

e) PEP carboxylase (orthophosphate : oxaloacetate carboxy-lyase (phosphorylating) EC 4.1.1.31): this was assayed in the presence of NADH and malate dehydrogenase by following the rate of incorporation of [^{14}C]-bicarbonate into malate (Lane et al., 1969). When required, acetyl CoA (0.4 μmol.ml^{-1}) was included as an activator

f) PEP transphosphorylase (pyrophosphate : oxaloacetate carboxy-lyase (phosphorylating) EC 4.1.1.38): the assay was as for PEP carboxylase only in the presence of 10 mM potassium phosphate bufrer, pH 6.8.

g) Pyruvate carboxylase (pyruvate : carbon dioxide ligase (ADP) EC 6.4.1.1): this was assayed by measuring the incorporation of [^{14}C]-bicarbonate into malate in the presence of NADH and malate dehydrogenase (Sahl and Truper, 1977). Acetyl CoA (0.4 μmol.ml^{-1}) was included in the assay as an activator.

h) Fructose-1,6-bisphosphatase (fructose-1,6-bisphosphate phosphohydrolase, EC 3.1.3.11): this was assayed by measuring the liberation of inorganic phosphate from fructose-1,6-bisphosphate with the method of Pontremoli (1966). Inorganic phosphate was estimated with the amidol reagent of Allen (1940).

i) Phosphoglycollate phosphatase (phosphoglycollate phosphohydrolase, EC 3.1.3.18): the method of Anderson and Tolbert (1966) was used, measuring the liberation of inorganic phosphate from sodium phosphoglycollate with the amidol reagent.

j) Hydroxypyruvate reductase (D-glycerate-NAD oxidoreductase, EC 1.1.1.29): this was assayed by following the oxidation of NADH at 340 nm (Large and Quayle, 1963). The reaction mixture (1.5 ml) contained 50 μmol phosphate buffer, pH 7.0; 0.2 μmol NADH; test protein. The reaction was initiated by the addition of 1 μmol lithium hydroxypyruvate.

k) 3-Hexulose phosphate synthase: this was assayed by measuring the incorporation of label from [^{14}C]-formaldehyde into sugar phosphates with D-ribose-5-phosphate as substrate (Lawrence et al., 1970). The reaction mixture (final volume 0.4 ml) contained 20 μmol phosphate buffer, pH 7.0; 2 μmol $MgCl_2.6H_2O$; 2 μmol ribose-5-phosphate; 2 μmol formaldehyde (specific activity 0.01 μCi μmol^{-1}). The reaction was started

by the addition of the test protein and stopped after 5 min with 1.5 ml of ethanol. Sugar phosphates were precipitated with 0.1 ml of 5% (w/v) barium acetate and radioactivity counted.

19. ASSAY OF MOLECULAR WEIGHT STANDARDS

a) Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) (Escherichia coli): activity was measured by the method of Garen and Levinthal (1960).

b) Alcohol dehydrogenase (alcohol : NAD^+ oxidoreductase, EC 1.1.1.1) (yeast): activity was measured by the method of Racker (1955).

c) Catalase (hydrogen-peroxide : hydrogen-peroxide oxidoreductase, EC 1.11.1.6) (bovine liver): activity was measured by the method of Chance and Maehly (1955).

d) Glutamate dehydrogenase (L-glutamate : NAD(P) oxidoreductase (deaminating), EC 1.4.1.2): activity was measured by the method of Fahien and Cohen (1970).

e) β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23): the assay was based on that of Miller (1972).

20. ENZYME UNITS

One unit of activity was defined as the amount of enzyme required to transform 1 μmol of substrate per minute.

21. MOLECULAR WEIGHT DETERMINATION BY GEL FILTRATION

The molecular weights of RuBP carboxylase from R. vannielii (RM5) and M. capsulatus (Bath) were determined by gel filtration on a column (1 x 90 cm) of Sephadex G200, equilibrated at 4°C with TEMMB buffer. Escherichia coli, β-galactosidase (mol. wt. 540,000), horse spleen apoferritin (mol. wt. 443,000), beef liver glutamate dehydrogenase (mol. wt. 320,000), bovine liver catalase (mol. wt. 232,000), yeast alcohol dehydrogenase (mol. wt. 141,000) and E. coli alkaline phosphatase (mol. wt. 86,000) were used as standards (Darnell and Klotz, 1975). A 0.5 ml sample containing enzyme and marker proteins was applied to the column, eluted at a flow rate of 10 $ml.h^{-1}$ and 1 ml fractions collected. Enzymes were located by assaying fractions for activity and apoferritin located by its absorbance at 412 nm.

22. SUCROSE DENSITY GRADIENT CENTRIFUGATION

Sucrose solutions for density gradient centrifugation were prepared in TEMMB buffer at concentrations from 0.2 to 0.8M. Unless otherwise indicated, linear 0.2 to 0.8M gradients or step gradients of equal volumes 0.2, 0.4, 0.6 and 0.8M sucrose were used (after Tabita and McFadden, 1974c). Gradients routinely had a total volume of 16 ml in 20 ml polycarbonate centrifuge tubes (Measuring and Scientific Equipment Ltd., Crawley, Sussex). Up to 25 mg of protein (1ml solution) was layered onto each gradient and overlaid with liquid paraffin. Centrifugation was donc at $4^{o}C$ in an MSE Superspeed 65 centrifuge with a pre-cooled MSE 8 x 25 titanium angle rotor. Gradients were centrifuged at 55,000 r.p.m. (240,000 g average) for 1 h 20 min at speed and stopped with the centrifuge brake on. Gradients were left in a vertical position for 10 min and then unloaded from the base of the tube using an MSE tube piercer. 1 ml fractions were collected and monitored for absorbance at 280 nm using 0.8M sucrose in TEMMB buffer as blank.

Certain gradients (56 ml total volume) were centrifuged at 23,500 r.p.m. (80,000 g average) for between 20 to 24 h using an MSE 3 x 70 aluminium swing-out rotor. Preparation and unloading of these gradients was as described above. Any other variation in centrifugation technique was as indicated in the Results section.

23. PURIFICATION OF R. VANNIELII (RM5) RuBP CARBOXYLASE

R. vannielii (RM5) RuBP carboxylase was purified from 50 l of culture grown on PM-medium and harvested in the late exponential phase of growth. All procedures were done at 4°C. 96 ml of crude soluble extract was slowly taken to 35% (w/v) saturation with $(NH_4)_2SO_4$ and stirred for 30 min. The precipitate was removed by centrifugation at 30,000 g for 20 min and discarded. The supernatant was then taken to 60% (w/v) saturation with $(NH_4)_2SO_4$ and stirred for 1 h. The pellet obtained after centrifugation (30,000 g for 20 min) was dissolved in TEMMB buffer (22 ml) and dialysed overnight against 200 volumes of the same buffer. The enzyme solution was then applied to a column (3.5 x 100 cm) of Ultrogel AcA 22 (LKB, Bromma, Sweden) equilibrated with TEMMB buffer. This was eluted at a flow rate of 10 $ml.h^{-1}$ and 5 ml fractions collected using an LKB Ultrorac Fraction Collector. Fractions with significant RuBP carboxylase activity were pooled and taken to 60% (w/v) saturation with $(NH_4)_2SO_4$ and stirred for 1 h. Following centrifugation at 30,000 g for 20 min the pellet was dissolved in TEMMB buffer (6 ml) and dialysed overnight against 300 volumes of the same buffer. The enzyme solution was then applied to a diethylaminoethyl (DEAE) cellulose column (2 x 10 cm) equilibrated with 20 mM Tris-HCl, pH 8.0 containing 5 mM $MgCl_2.6H_2O$ and 5 mM 2-mercaptoethanol (TMM-buffer). The column was eluted under gravity at a flow rate of

50 ml.h^{-1} by a linear 0 to 1M NaCl gradient prepared in TMM buffer. Active fractions were pooled and concentrated in a 10 ml ultrafiltration cell (Amicon, High Wycombe, Bucks) over an Amicon 'Diaflo' PM10 membrane. The resultant enzyme solution (2 ml) was finally loaded onto a 0.2 to 0.8M discontinuous sucrose gradient (56 ml total volume). After centrifugation at 80,000 g for 24 h the gradient was fractionated and active fractions pooled and concentrated by ultrafiltration.

24. PARTIAL PURIFICATION OF R. RUBRUM RuBP CARBOXYLASE

R. rubrum RuBP carboxylase was partially purified using DEAE cellulose column chromatography and sucrose density gradient centrifugation. All procedures were done at 4^{o}C.

5 ml of crude soluble extract was applied to a DEAE column (2 x 10 cm) equilibrated with TMM buffer. The column was washed through with TMM buffer until all 280nm absorbing material had been removed and then eluted at a flow rate of 50 ml.h^{-1} by a linear gradient of 0 to 0.5M NaCl in TMM buffer (total volume 500 ml). 5 ml fractions were collected and those with significant RuBP carboxylase activity were pooled and concentrated by ultrafiltration over a PM10 membrane.

The enzyme solution (0.9 ml) was then loaded onto a linear 0.2 to 0.8M sucrose gradient, centrifuged for 1 h 20 min at 240,000 g, fractionated and each fraction assayed for enzyme activity.

25. ENZYME PURIFICATIONS FROM M. CAPSULATUS (BATH)

a) Fractionation of crude extracts

The preliminary step in the purification of all enzymes from M. capsulatus (Bath) was the separation of soluble (S_{160}) extracts by DEAE column chromatography into four fractions (Colby and Dalton, 1978). This was done at 4°C. Crude soluble extract was applied to a 4.5 x 12 cm column of DEAE cellulose equilibrated with 20 mM Tris-HCl buffer, pH 7.0. Material not binding to the column (fraction A) was eluted with 20 mM Tris-HCl buffer, pH 7.0. The column was then eluted with successive 500 ml batches of the same buffer containing respectively 0.1M, 0.2M (yielding fraction B) and 0.5M (yielding fraction C) NaCl.

Each of the four fractions was immediately frozen by dropwise addition to liquid nitrogen and stored at -80°C.

b) Purification of M. capsulatus (Bath) RuBP carboxylase

All procedures were done at 4°C. 21 ml of fraction B was concentrated by ultrafiltration over a PM10 membrane to a volume of 1.95 ml. A sample of this enzyme solution (0.6 ml) was applied to a column (1 x 90 cm) of Sephadex G200, eluted at a flow rate of 10 $ml.h^{-1}$ with TEMMB buffer and 1.5 ml fractions collected. Those with significant RuBP carboxylase activity were pooled and concentrated by ultrafiltration over a PM10 membrane. 0.6 ml of this enzyme solution was loaded onto a 0.2 to 0.8M linear sucrose gradient, centrifuged for 1 h 30 min at 240,000 g and fractionated. Those fractions with significant RuBP carboxylase activity were combined, dialysed overnight against 500 volumes of TEMMB buffer and concentrated by ultrafiltration over a PM10 membrane. This enzyme solution (0.4 ml) was centrifuged in a second 0.2 to 0.8M linear sucrose gradient at 240,000 g for 1 h 20 min and fractionated.

c) Partial purification of M. capsulatus (Bath) hydroxypyruvate reductase

Hydroxypyruvate reductase was partially purified by applying 30 ml of fraction A to an Ultrogel AcA 34 column (6.3 x 60 cm), and eluting at a flow rate of 50 $ml.h^{-1}$ with Tris-HCl, pH 7.0. This was done at 4°C. 8 ml fractions were collected and those with significant enzyme activity were pooled and frozen in liquid nitrogen.

d) Partial purification of M. capsulatus (Bath) phosphoglycollate phosphatase

Phosphoglycollate phosphatase was partially purified by centrifugation of fraction B into a 0.2 to 0.8M sucrose gradient.

26. RADIOTRACER STUDIES

a) Products of cell-free carbon dioxide fixation

To determine products of carbon dioxide fixation by soluble extracts or partially purified RuBP carboxylase, of M. capsulatus (Bath), the assay was done using [^{14}C]-bicarbonate at a specific activity of 8 μCi μmol^{-1}. Radioactive compounds formed were identified by chromatography and autoradiography.

b) Labelling and extraction of pool metabolites

Chemostat grown cells of M. capsulatus (Bath) were harvested by centrifugation (10,000 g for 10 min), then washed and resuspended in 10 ml of AMS-medium to a cell density of either 10 mg.ml^{-1} (for [^{14}C]-bicarbonate incorporation) or 5 mg.ml^{-1} (for [^{14}C]-I-glycollate incorporation). The suspension, in a 50 ml round bottomed flask sealed with a rubber serum cap,

was aerated by stirring with a magnetic follower and partially submerged in a water bath at the required temperature. After equilibration for 5 min, 10 ml of methane was injected into the flask, followed after 10 min by the [^{14}C] substrate (200 μCi of [^{14}C]-bicarbonate or 50 μCi [^{14}C]-I-glycollate). At various time intervals after addition of label, a 1 ml sample was removed by hypodermic syringe and added to 4 ml of hot (60°C) absolute ethanol. The resulting precipitate was removed by centrifugation and further extracted with two changes of 50% (v/v) hot ethanol. The supernatants were combined and the volume reduced to 0.5 ml in a Vacuum Rotary Evaporator (Quickfit Instrumentation, England).

c) Chromatography and autoradiography

Radioactive compounds were separated by two dimensional ascending paper chromatography. An aliquot (normally 25 μl) of labelled extract was applied to the origin of a sheet of 25 x 25 cm Whatman No.1 chromatography paper and a number of such papers fitted to a universal frame. Chromatograms were developed overnight in the first direction with a phenol : water : glacial acetic acid : ethylenediamine-tetracetic acid (IM) (840 : 149 : 10 : 1) solvent system and after drying at room temperature in a stream of air, were developed overnight in the second direction in equal volumes of propionic

acid : water (620 : 790) and n-butanol : water (1246 : 84). The position of labelled metabolites was determined by exposing the chromatograms to 'Kodirex' X-ray film for up to one month. The radioactivity per metabolite was measured by cutting out radioactive spots and counting them in the triton-toluene scintillation system. Radioactive spots from duplicate chromatograms were cut out, eluted with 50% (v/v) ethanol and the labelled metabolite re-chromatographed in the same solvent system with a series of standards. Amino acid standards were detected by spraying with 0.25% (w/v) ninhydrin in acetone, carboxylic acids with 0.1% (w/v) bromocresol green (pH 5.0) in ethanol and phosphorylated compounds with 60% (w/w) perchloric acid, 5 ml; 1M HCl, 10 ml; 4% (w/v) ammonium molybdate, 25 ml; distilled water, 60 ml followed by exposure to ultra violet light (Bandurski and Axelrod, 1951). Metabolites were positively identified when they co-chromatographed with the authentic standard.

The radioactivity per metabolite was expressed either as a percentage of the total radioactivity in that particular sample or as c.p.m. (mg dry wt. of cells)$^{-1}$.

SECTION III

RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE AND CARBON DIOXIDE FIXATION IN *RHODOMICROBIUM VANNIELII* (RM5)

1. INTRODUCTION

R. vannielii (RM5) exhibits a polymorphic vegetative cell cycle in which three distinct cell types are expressed (Dow, 1974). The cell cycles of organisms within the *Rhodospirillaceae* range in complexity from that of *Rhodomicrobium* to the simple monomorphic cell cycle of *R. rubrum*. It is possible to arrange these into a gradient of complexity which may be indicative of morphogenetic evolution (Fig. 12; Whittenbury and Dow, 1977).

It has been shown by France (1978), that as an alternative to the complex cell cycle characteristic of *Rhodomicrobium*, *R. vannielii* (RM5) may also replicate by a simplified vegetative cell cycle not involving the formation of chains of cells (Fig. 13). Although the control of these two forms of vegetative cell cycle is poorly understood, evidence does suggest that there may be an environmental switch between complex and simplified cycles, possibly mediated by light intensity and/or carbon dioxide tension (France, 1978; C. S. Dow personal communication). The work presented in this thesis has been done with the organism expressing the simplified cell cycle.

The limited information that is available regarding RuBP carboxylase and carbon dioxide fixation in the *Rhodospirillaceae* has already been indicated in the General

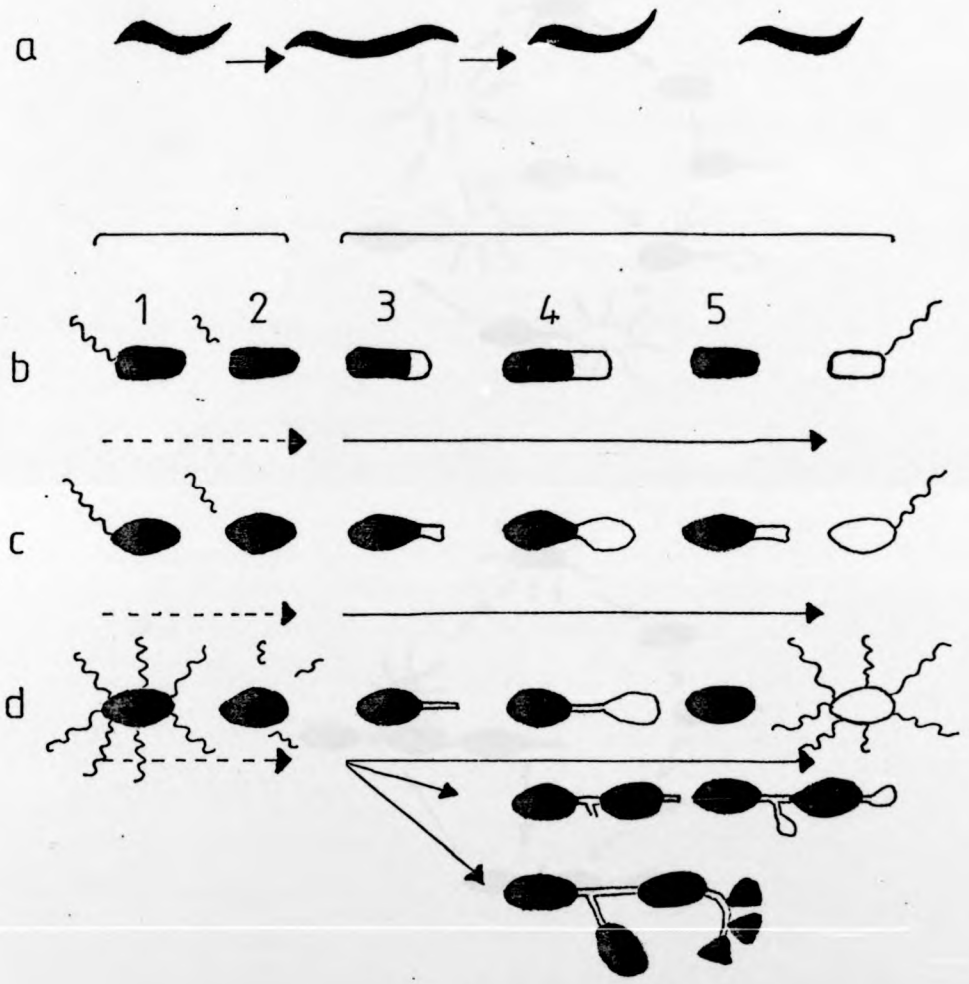


Fig. 12 Rhodospirillaceae ordered in degrees of morphological/cell cycle complexity

(a) R. rubrum; (b) R. acidophila;

(c) R. palustris, R. viridis;

(d) Rhodomicrobium sps

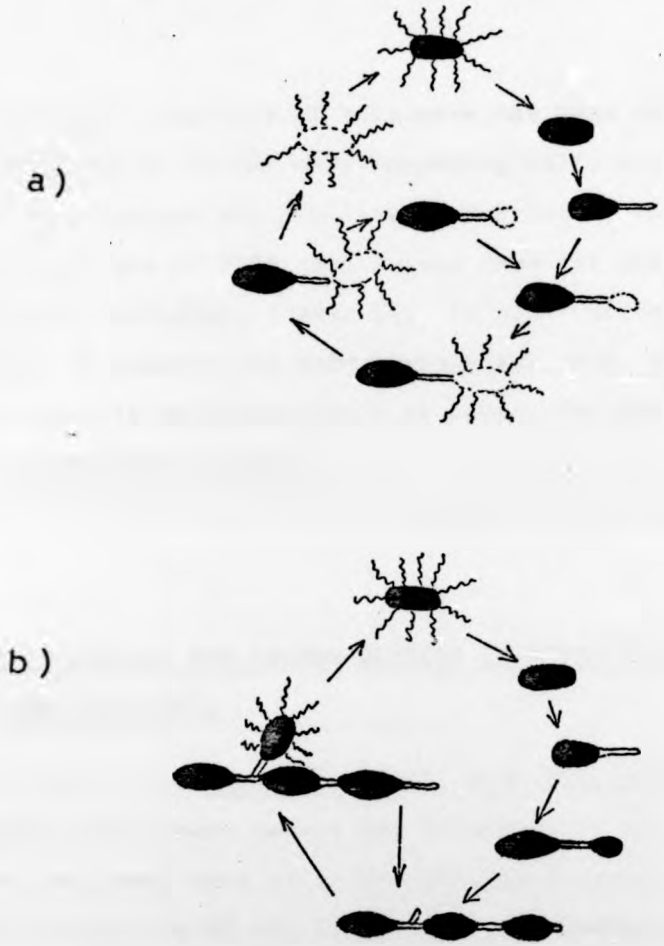


Fig. 13 Simplified (a) and complex (b) cell cycles of R. vannielii (RM5)

Introduction. The bulk of this work has been done with R. rubrum, which is not only morphologically very simple but also possesses the smallest, and probably the most primitive, form of RuBP carboxylase thus far characterised (Tabita and McFadden, 1974a, b). It was therefore of interest to examine the RuBP carboxylase of R. vannielii (RM5) which is morphologically at least, the most complex of the Rhodospirillaceae.

2. REQUIREMENTS FOR CARBON DIOXIDE FIXATION BY RHODOMICROBIUM VANNIELII (RM5)

Whole cells of R. vannielii (RM5), when resuspended in complete (PM) growth medium and incubated in the light, exhibit a linear rate of carbon dioxide fixation over the period from 10 to 60 min (Fig. 14). Photoheterotrophically grown cells taken from a chemostat (dilution rate of 0.024 h^{-1}) were used for this experiment. In the absence of light and consequently energy supply, carbon dioxide fixation was negligible. Furthermore, cells resuspended in basal salts did not show as high a rate of carbon dioxide fixation as those resuspended in PM-medium. The results shown in Figure 14 were taken from a single experiment and it became evident over a number of such experiments, that

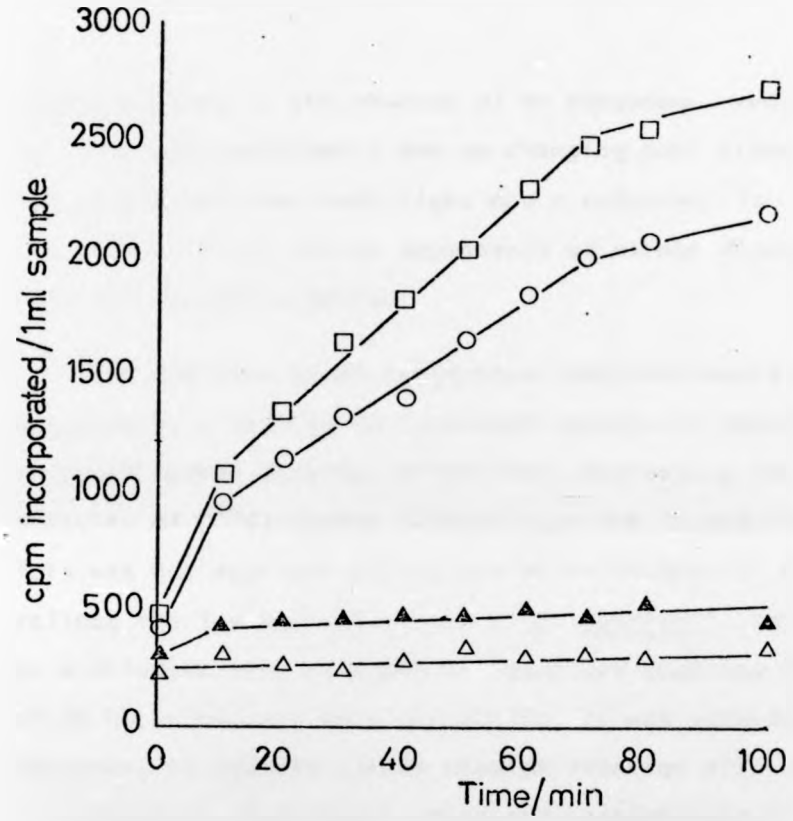


Fig. 14 Incorporation of [^{14}C]-carbon dioxide by intact cells of R. vannielii (RM5)

□–□ PM medium : light

○–○ Basal salts : light

▲–▲ PM medium : dark

△–△ PM medium : light : killed cells

fixation rates in the absence of an exogenous reductant were variable, presumably due to changing pool sizes. The requirement for both light and a reductant is indicative of the energy dependence of carbon dioxide fixation in this organism.

The addition of an exogeneous reductant would be expected to give rise to increased amounts of endogen ously produced carbon dioxide, effectively decreasing the specific activity of [^{14}C]-carbon dioxide over the incubation period. This was not apparent during one hours incubation which may reflect the low metabolic rate of R. vannielii (RM5) grown at a dilution rate of 0.024 h^{-1} (culture doubling time, td, of 29 h). However, as a precaution, it was considered necessary to measure carbon dioxide fixation with cells resuspended in both basal salts and complete growth medium.

The initial large increase in the amount of carbon dioxide apparently fixed between zero and 10 min, was a consistently observed phenomenon for which no satisfactory explanation has been found. It may possibly be due to the transient elevated specific activity of bicarbonate on addition of label owing to equilibration of carbon dioxide between its various forms (11).

(11)

$$CO_2 + H_2O \underset{}{\overset{\text{Kdiss } 5.71 \times 10^{-7}}{\rightleftharpoons}} H^+ + HCO_3^- \overset{\text{Kdiss } 4.8 \times 10^{-11}}{\rightleftharpoons} H^+ + CO_3^{2-}$$

(dissociation constants quoted at 30°C, from: Handbook of Chemistry and Physics, ed. R. C. Weast, published by The Chemical Rubber Co., Ohio, USA).

Bicarbonate (HCO_3^-) has been shown to be the ionic species probably transported into R. rubrum (Christeller and Laing, 1978). The addition of label, which at a pH of 8.2, would be predominantly [^{14}C]-HCO_3^-, will give an initially high specific activity of the species of carbon dioxide that is most likely taken up by the cell, although rapid equilibration will occur. This effect may be sufficient to give the observed early high rate of carbon dioxide fixation.

The calculated specific activity of 0.23 μmol carbon dioxide fixed.h^{-1}.(mg.dry wt of cells)$^{-1}$, is comparable to that reported for photoheterotrophically grown R. rubrum (Slater and Morris, 1973a). However, as will be shown later, the rate of carbon dioxide fixation was found to be dependent upon growth conditions.

3. THE KEY ENZYMES OF THE CALVIN CYCLE IN RHODOMICROBIUM VANNIELII (RM5)

Carbon dioxide fixation by cell-free extracts of R. vannielii (RM5) was investigated using RuBP and its immediate precursors in the Calvin cycle as potential carboxylation substrates. The effects of β-NADH and/or ATP additions, on the rates of carbon dioxide fixation were also examined (Table 3). The extract was prepared from cells grown as a batch culture on PM-medium.

Carbon dioxide fixation was stimulated by RuBP indicating the presence of RuBP carboxylase in R. vannielii (RM5). With both ribulose-5-phosphate (Ru5P) and ribose-5-phosphate (R5P) as test substrates, ATP and β-NADH were required for maximal activity. The ATP requirement is in agreement with both Ru5P and R5P requiring conversion to RuBP by a phosphoribulokinase before carbon dioxide fixation can occur. The decreased activity of the carboxylase when assayed using Ru5P or R5P as test substrate, indicates that the kinase either has low activity or requires specific activation. Although commercial preparations of Ru5P have been shown to contain an inhibitor of 3-hexulose phosphate synthase (Kemp, 1972), were this also affecting phosphoribulokinase, increased activity would have resulted from the use of R5P rather than Ru5P as substrate. This was not the case. Furthermore,

ADDITIONS	CO_2 FIXATION ($nmol\ CO_2$ fixed. $min^{-1}.mg.protein^{-1}$)
none	0.2
ATP	0.3
β-NADH	0.2
ATP + β-NADH	0.4
Ribulose 1,5-P_2	53.3
Ribulose 1,5-P_2 + ATP	57.3
Ribulose 1,5-P_2 + β-NADH	54.0
Ribulose 1,5-P_2 + ATP + β-NADH	57.5
Ribulose 5-P	1.2
Ribulose 5-P + ATP	25.7
Ribulose 5-P + β-NADH	1.5
Ribulose 5-P + ATP + β-NADH	40.0
Ribose 5-P	0.5
Ribose 5-P + ATP	7.0
Ribose 5-P + β-NADH	0.4
Ribose 5-P + ATP + β-NADH	22.5

Table 3 Carbon dioxide fixation by the soluble fraction of cell-free extracts of R. vannielii (RM5)

preincubation of R5P with purified yeast phosphoriboisomerase prior to starting the reaction, did not bring about any increase in the amount of carbon dioxide fixed, indicating that the isomerase was not rate limiting. In addition, no appreciable kinase activity was associated with the particulate fraction of cell-free extracts.

Enhancement of phosphoribulokinase activity by β-NADH has been reported for both Rhodopseudomonas spheroides (Rindt and Ohmann, 1969) and Nitrobacter winogradskyi (Kiesow et al., 1977). This effect was further investigated in R. vannielii (RM5) using increasing concentrations of β-NADH with Ru5P (and ATP) as test substrate (Fig. 15). Unlike R. spheroides and N. winogradskyi, an absolute requirement for β-NADH was not observed there being appreciable kinase activity in its absence. This may however, be due to β-NADH being present in cell-free extracts, possibly enzyme bound. Further investigation of this necessitates studies on the purified enzyme.

The presence of RuBP carboxylase, phosphoribulokinase and phosphoriboisomerase in cell-free extracts of R. vannielii (RM5) indicates that the Calvin cycle probably functions in this organism. This would be in accordance with the energy dependence of carbon dioxide fixation by whole cells. The results have also indicated that phosphoribulokinase may be a site of regulation of carbon dioxide fixation based upon the intracellular concentration of β-NADH.

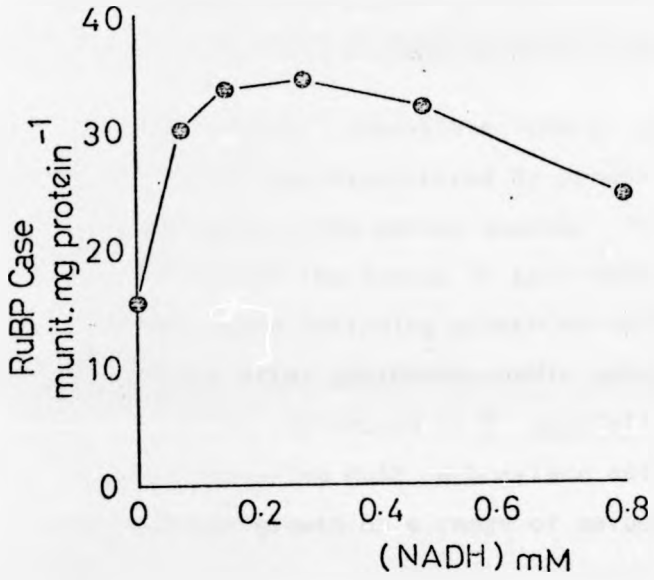


Fig. 15 The effect of β-NADH on ribulose 5-phosphate : ATP dependent carbon dioxide fixation by the soluble fraction of R. vannielii (RM5) cell-free extract

4. EFFECT OF CARBON SOURCE ON THE LEVEL OF RuBP CARBOXYLASE IN CELL-FREE EXTRACTS OF RHODOMICROBIUM VANNIELII (RM5)

The purification of RuBP carboxylase from R. rubrum (Tabita and McFadden, 1974a) was facilitated by growth of the organism on butyrate as the carbon source. This resulted in a specific activity of the enzyme in cell-free extracts, far higher than that found following growth on malate and comparable to that after photoautotrophic growth. Whether this effect could be reproduced in R. vannielii (RM5) was investigated by measuring RuBP carboxylase activity in cell-free extracts after growth on a range of carbon substrates (Table 4).

The highest specific activity was found in extracts of malate grown cells with comparatively low activity in extracts of cells grown on the more reduced carbon substrates. Furthermore, extracts of photoautotrophically grown cells also had a low specific activity. Growth of R. vannielii (RM5) was very poor both autotrophically and on the more reduced carbon substrates, in comparison to that on malate and other tricarboxylic acid cycle intermediates. It is not improbable, in view of the energy dependence of the Calvin cycle, that synthesis of RuBP carboxylase is regulated by the availability of reducing power, and the utilisation of poorly metabolised substrates may not provide sufficient reducing power to sustain enzyme synthesis. Alternatively, the role of RuBP

Carbon Growth Substrate	Specific Activity m.unit.mg.protein^{-1}
sodium pyruvate	19.3
sodium pyruvate + CO_2	19.1
sodium malate	39.2
sodium succinate	25.6
10 mM sodium pyruvate + 10 mM sodium malate	25.7
ethanol + CO_2	20.8
sodium butyrate + CO_2	16.8
sodium propionate + CO_2	16.7
sodium acetate + CO_2	13.1
sodium lactate + CO_2	15.3
hydrogen + CO_2	16.0

Table 4

The effect of growth substrate on the specific activity of RuBP carboxylase in crude cell extracts of R. vannielii (RM5)

carboxylase in transforming carbon dioxide into a form which can act as an hydrogen acceptor (Lascelles, 1960), may be of little importance in Rhodomicrobium and during growth on butyrate, ethanol, etc., other methods adopted as outlets for reducing power. On the assumption that the in vitro enzyme activity is a reflection of enzyme synthesis, it is clear that RuBP carboxylase synthesis is not substantially repressed during growth on malate. The specific activity of 39.2 m unit.mg protein^{-1} in extracts of malate grown cells is some 5-fold greater than that reported for malate grown R. rubrum (Tabita and McFadden, 1974a) and this may be indicative of a different mode of regulation.

5. PURIFICATION OF RHODOMICROBIUM VANNIELII (RM5) RuBP CARBOXYLASE

The possibility of using a one-step purification technique (Goldthwaite and Bogorad, 1971) was investigated but this did not result in an electrophoretically pure protein (see page 148). A conventional purification procedure was then adopted, the protocol for which is shown in Table 5, and described in the Materials and Methods section (subsection 23).

Table 5 Purification protocol for *R. vannielii* (RM5) RuBP carboxylase

Step	protein (mg)	activity (units)	specific act. units.mg protein^{-1}	yield (%)
10,000 g (10 min) supernatant	2100	56.7	0.027	-
120,000 g (1½ h) supernatant	1008	50.4	0.050	88.9
35-60% (w/v) SAT. $(NH_4)_2SO_4$ ppt.	446	37.5	0.084	66.1
gel filtration on Ultrogel AcA22 Pooled and conc. active fractions	49.4	28.3	0.57	49.7
DEAE cellulose chromatography Pooled and conc. active fractions	14.0	21.0	1.5	37.0
0.2-0.8 M sucrose gradient	3.9	7.4	1.9	13.0

The crude cell extract was prepared from approximately 12 g dry wt. of cells. The procedure gave a 13% yield and an overall 70-fold purification of the enzyme. Therefore, the protein only constituted about 2.6% (w/w) of the total soluble protein present in the 120,000 g supernatant.

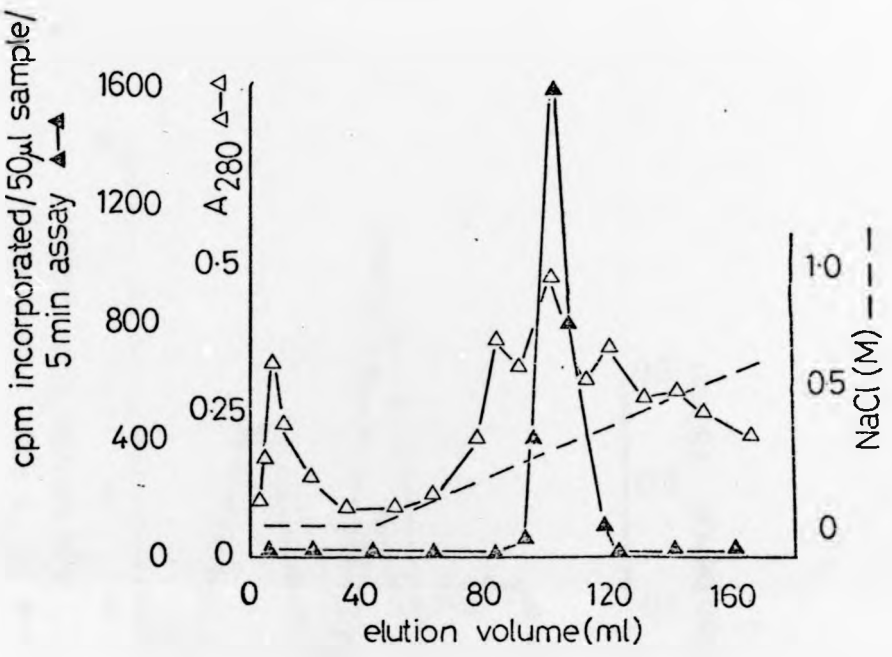
Enzyme eluted from both Ultrogel AcA22 (Fig. 16) and DEAE cellulose (Fig. 17) columns as a single peak of activity indicating, on the basis of both molecular size and overall charge, the presence of only one form of RuBP carboxylase in R. vannielii (RM5). Following sucrose density gradient centrifugation (Fig. 18) the enzyme was judged to be pure by it moving as a single band during electrophoresis on gels polymerised from 4 to 7.5% (w/v) acrylamide (Fig. 19). This technique lessens the possibility of there being contaminants present having similar charge but slightly different molecular weights (Hedrick and Smith, 1968).

The specific activity of the pure protein, 1.9 units. mg protein^{-1}, is similar to that reported for plant RuBP carboxylases although somewhat higher than that of most bacterial RuBP carboxylases

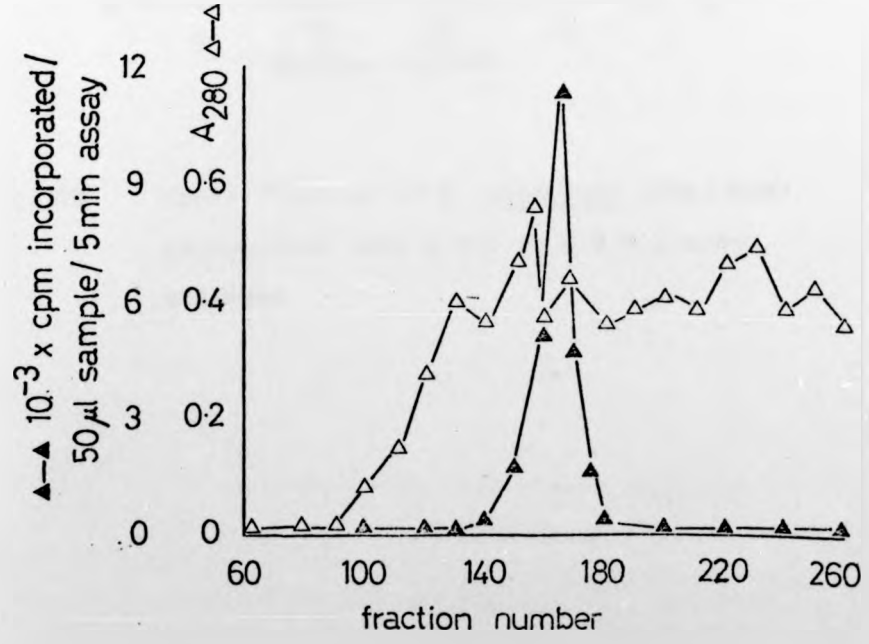
Fig. 17 DEAE cellulose chromatography of RuBP carboxylase from R. vannielii (RM5)

Fig. 16 Elution of R. vannielii (RM5) RuBP carboxylase from a column of Ultrogel AcA 22

of RuBP
(RM5)



RuBP
Ultrogel



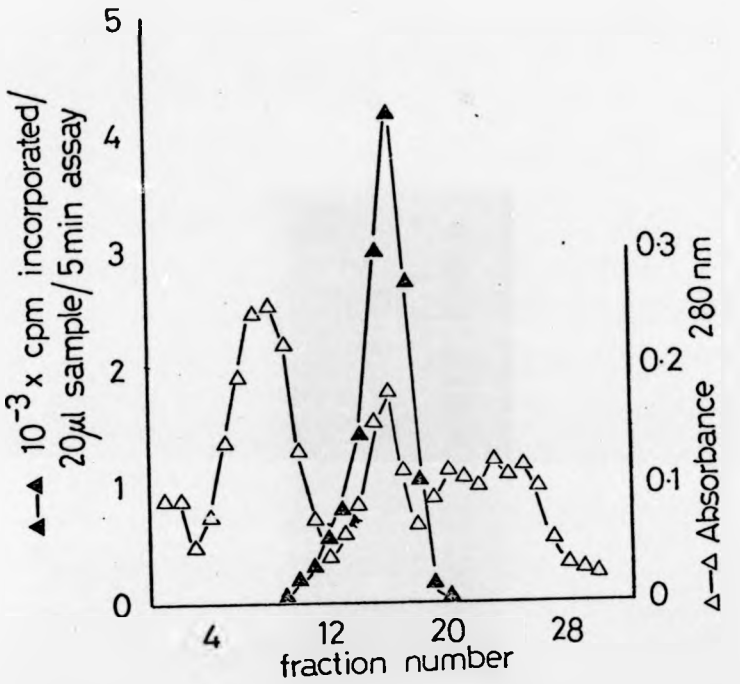


Fig. 18 Centrifugation of R. vannielii (RM5) RuBP carboxylase into a 0.2 to 0.8 M sucrose gradient

Fig. 19 Polyacrylamide gel electrophoretograms of R. vannielii (RM5) RuBP carboxylase.

A 50 μg sample was applied to each gel polymerised from (left to right) 7.5, 6, 5, 4 and 4.5% (w/v) acrylamide.

6. PROPERTIES OF THE PURIFIED RuBP CARBOXYLASE FROM RHODOMICROBIUM VANNIELII (RM5)

a) Stability of RuBP carboxylase

The purified enzyme was fully stable for up to one month when stored at -80°C. After storage at both 4°C and -20°C, the enzyme had lost approximately 50% of its original activity after 21 days. When buffer not containing 2-mercaptoethanol was used in enzyme purification, aggregates of RuBP carboxylase was readily formed which could be separated by electrophoresis on a range of concentrations of acrylamide gels (Fig. 20). This effect is widespread amongst plant RuBP carboxylases (R. J. Ellis, personal communication) and may be a cause of some of the contradictory reports regarding the bacterial enzyme which have been discussed in the General Introduction.

b) Molecular Weight

The molecular weight of the RuBP carboxylase was measured by gel filtration on Sephadex G200 as described in the Materials and Methods Section. This gave a value of 430,000 (Fig. 21) The division of RuBP carboxylases into three classes on the basis of molecular weight (Anderson *et al.*, 1968) has already been indicated. On this basis, the *R. vannielii* (RM5) enzyme falls between the intermediate class (mol.wt. 240-360,000) and the large class (mol.wt. approx. 500,000).

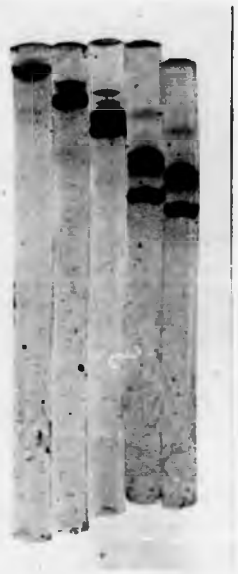


Fig. 20 Polyacrylamide gel electrophoretograms of R. vannielii (RM5) RuBP carboxylase purified in the absence cf 2-mercaptoethanol.

A 50 μg sample was applied to each gel polymerised from (left to right) 7.5, 6, 5, 4.5 and 4% (w/v) acrylamide.

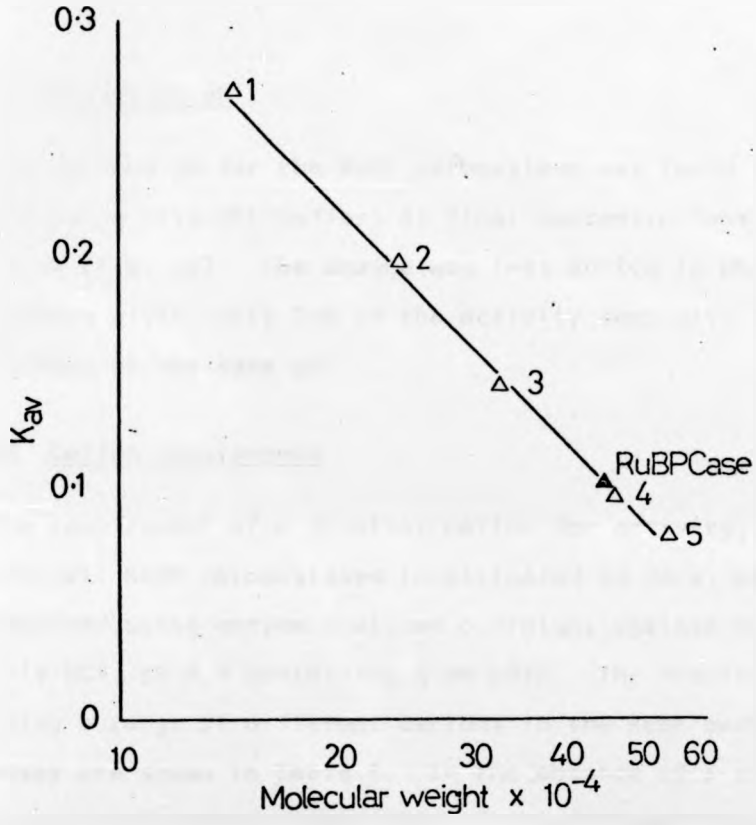


Fig. 21 Molecular weight estimation of R. vannielii (RM5) RuBP carboxylase on a Sephadex G200 column

Molecular weight standards:

(1) alcohol dehydrogenase; (2) catalase;

(3) glutamate dehydrogenase; (4) ferritin;

(5) β-galactosidase

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

V_e - elution volume
V_t - bed volume
V_o - void volume

c) Effect of pH

The optimum pH for the RuBP carboxylase was found to be 8.2 using Tris-HCl buffers at final concentrations of 60 mM (Fig. 22). The enzyme was less active in phosphate buffers giving only 25% of the activity seen with Tris-HCl buffers at the same pH.

d) Cation requirement

The requirement of a divalent cation for activity, seen with all RuBP carboxylases investigated to date, was examined using enzyme dialysed overnight against 20 mM Tris-HCl, pH 8.0 containing 5 mM EDTA. The results obtained using a range of different cations in the RuBP carboxylase assay are shown in Table 6. In the absence of a cation, enzyme activity was negligible. Activity was restored with the inclusion of Mg^{2+} in the assay. The reaction was not however specific for Mg^{2+}, with Mn^{2+}, Co^{2+} and Ni^{2+}, each at a final concentration of 10 mM, giving 32, 39 and 133% respectively, of the activity seen with Mg^{2+}. The substitution of Mg^{2+} by Ni^{2+} and Co^{2+} has been shown with the plant RuBP carboxylase, to depend to some extent on the age and purity of the enzyme preparation (Kawashima and Wildman, 1970). However, the high activity observed with Ni^{2+} is of interest, particularly in view of the specificity of the *R. rubrum* RuBP carboxylase for Mg^{2+} (Tabita and McFadden, 1974a).

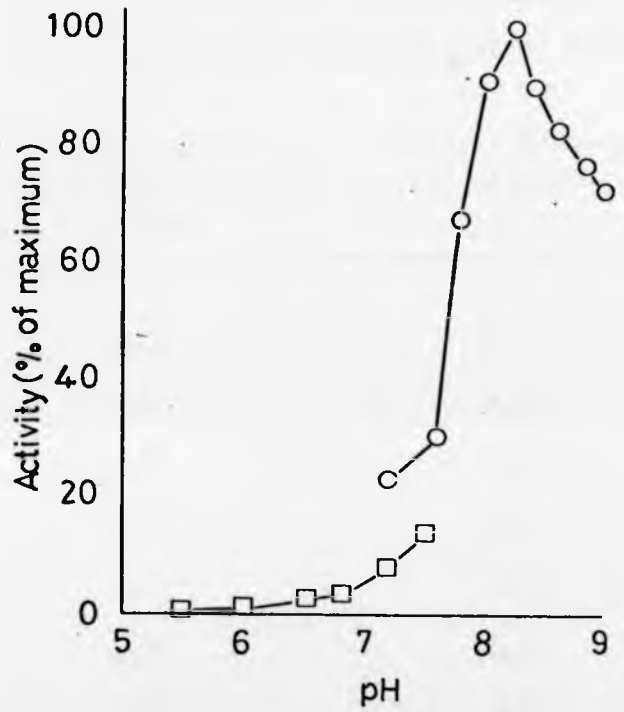


Fig. 22 The effect of pH on R. vannielii (RM5) RuBP carboxylase activity.

The maximum specific activity was 1.9 units.mg protein^{-1}.

□ - □ phosphate buffer

o - o Tris-HCl buffer

Table 6 The effect of various cations on the activity of purified R. vannielii (RM5) RuBP carboxylase

Cation in Assay Final Concentration of 10mM	Specific Activity (units.mg.protein^{-1})	% Activity with Mg^{2+}
none	0.34	21
Mg^{2+}	1.62	100
Co^{2+}	0.64	39
Ni^{2+}	2.15	133
Zn^{2+}	0.20	12
Mn^{2+}	0.52	32
Ca^{2+}	0.19	12
Fe^{2+}	0.32	20
Mo^{2+}	0.38	23
Cu^{2+}	0.13	8
Al^{2+}	0.13	8
Cd^{2+}	0.16	10

(1 unit = 1 μmol CO_2 fixed.min^{-1})

This may indicate that the conformation of the active site and the reaction mechanism, of the R. vannielii (RM5) carboxylase may be more closely related to plant RuBP carboxylases than to that of the R. rubrum enzyme.

Double reciprocal plots of specific activity against Mg^{2+} concentration for the R. vannielii (RM5) RuBP carboxylase gave a K_m value of 50 μM. This is much lower than the values obtained for spinach (1.0 mM; Wishnick et al., 1970), Pseudomonas facilis (1.4 mM; Kuehn and McFadden, 1969), R. rubrum (0.21 mM; Tabita and McFadden, 1974b), Thiobacillus intermedius (3.6 mM; Purohit et al., 1976) and Aphanocapsa 6308 (0.35 mM; Codd and Stewart, 1977) although similar to the 20 μM reported for Thiobacillus.A2 (Charles and White, 1976a).

e) Substrate concentration

Double reciprocal plots of specific activity against increasing RuBP concentration between 10 and 200 μM, gave a K_m for RuBP of 40 μM which is in agreement with the reported values of 14.8 μM for Thiobacillus novellus (McCarthy and Charles, 1975), 76 μM for T. intermedius (Purohit et al., 1976) and 53 μM for R. rubrum (Tabita and McFadden, 1974b). The problems associated with measuring the K_m for carbon dioxide have been discussed in the General Introduction. However, double reciprocal plots of specific activity against total carbon dioxide

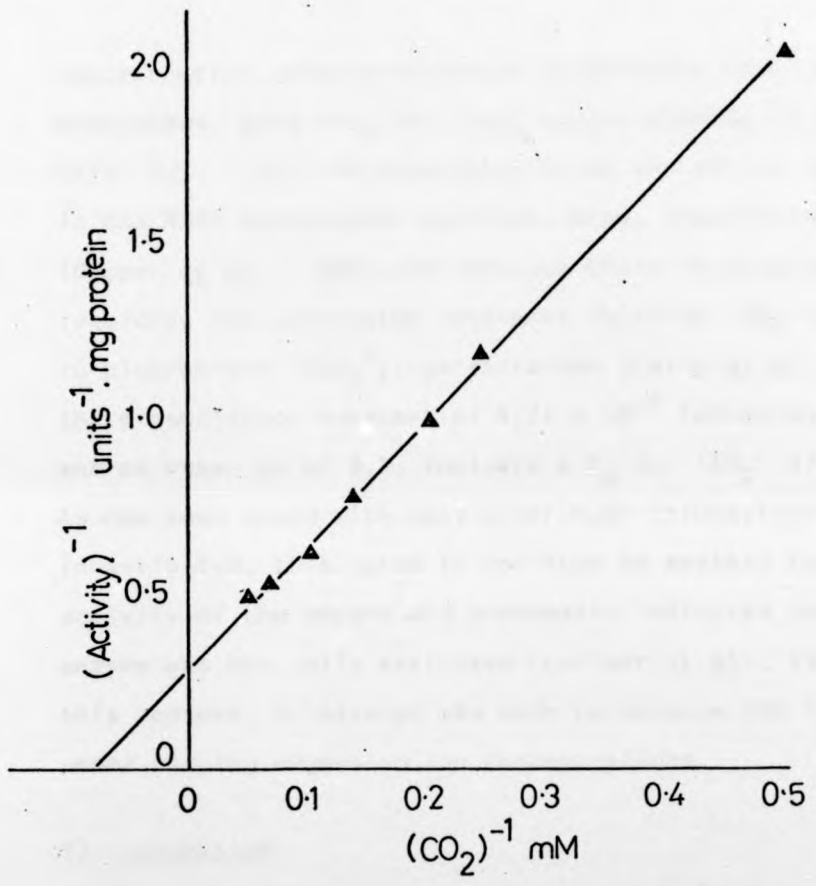


Fig. 23 Effect of carbon dioxide concentration on R. vannielii (KM5) RuBP carboxylase activity. Determination of K_m (carbon dioxide)

concentration, when provided as bicarbonate under an air atmosphere, gave a K_m for total carbon dioxide of 14.3 mM (Fig. 23). 'CO_2' is considered to be the active species in the RuBP carboxylase reaction rather than bicarbonate (Cooper et al., 1969) and although there is uncertainty regarding the conversion constants relating 'CO_2' concentration to bicarbonate (HCO_3^-) concentration (Laing et al., 1975), the dissociation constant of 4.71×10^{-7} (equation, page 83) and an assay pH of 8.2, indicate a K_m for 'CO_2' of 0.22 mM. As has been found with many other RuBP carboxylases investigated, this value is too high to explain the in vivo activity of the enzyme and presumably indicates that the enzyme was not fully activated (Lorimer et al., 1976). In this context, no attempt was made to measure the K_m for 'CO_2' under varying magnesium ion concentrations.

f) Inhibition

The inhibition of RuBP carboxylase by 6-phosphogluconate (6PG) has been discussed in the General Introduction as both a possible in vivo regulatory mechanism for carbon dioxide fixation and as a potential means of classification. When included in the preincubation mixture, prior to initiation of the reaction with RuBP, 6PG was found to be an effective inhibitor of the R. vannielii (RM5) RuBP carboxylase (Fig. 24). Only 50% of original activity was evident in the presence of

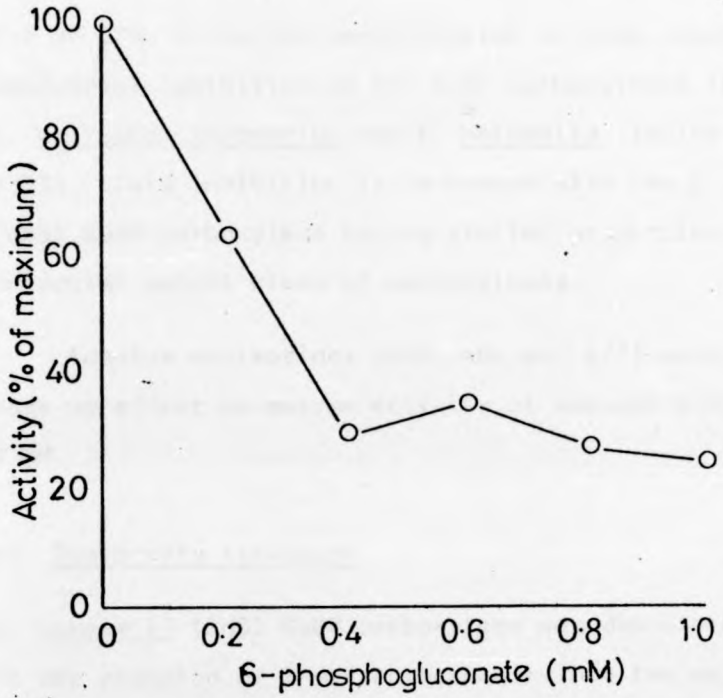


Fig. 24 Effect of 6-phosphogluconate on the activity of R. vannielii (RM5) RuBP carboxylase. The maximum activity was 1.9 units.mg protein^{-1}

0.3 mM 6PG, a similar concentration to that required for equivalent inhibition of the RuBP carboxylases from spinach, A. eutropha, Chromatium and E. halophila (Tabita and McFadden, 1972). This inhibition is in accord with the R. vannielii (RM5) RuBP carboxylase having similar properties to the large molecular weight class of carboxylases.

Adenine nucleotides (AMP, ADP and ATP) were found to have no effect on enzyme activity at concentrations up to 1 mM.

g) Quaternary structure

R. vannielii (RM5) RuBP carboxylase was dissociated with SDS in the presence of 2-mercaptoethanol into two major protein bands as revealed by electrophoresis on slab gels polymerised from 10% (w/v) acrylamide. These bands correspond to the large and small subunits. The large subunit was partially resolved by this gel system, into two individual bands. The mobility of the polypeptide subunit species in relation to the migration of dissociated protein standards, gave molecular weights of the three subunits as 56,200, 53,300 and 15,700 (Fig. 25).

The apparent heterogeneity of the large subunit was further investigated using SDS gels polymerised from an exponential gradient of 7.5 to 20% (w/v) acrylamide. The

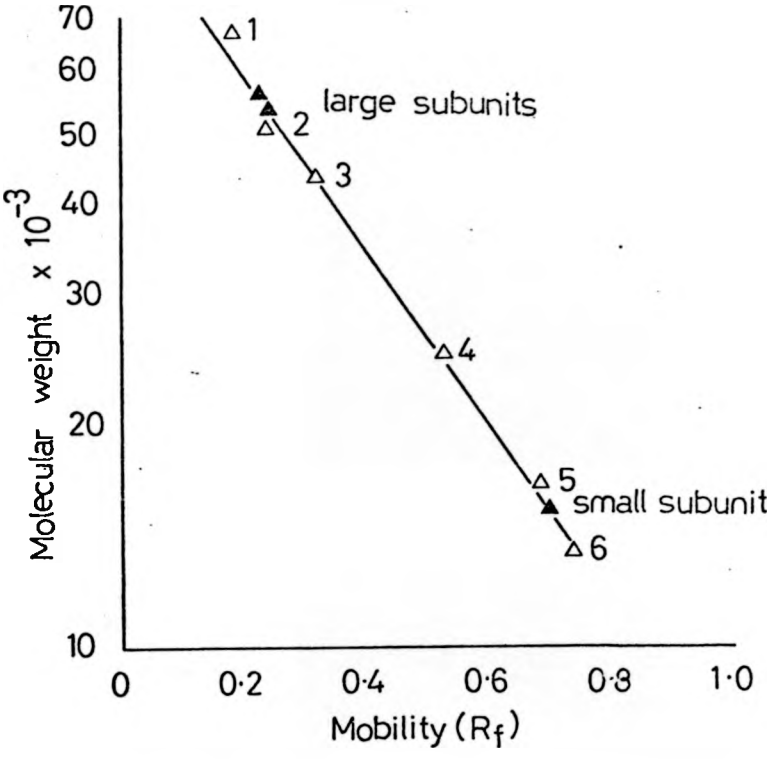


Fig. 25 SDS polyacrylamide gel electrophoresis (10% w/v gel) of R. vannielii (RM5) RuBP carboxylase

Marker proteins were

(1) bovine plasma albumin; (2) & (4) γ-globulins;.

(3) ovalbumin; (5) myoglobin; (6) cytochrome C

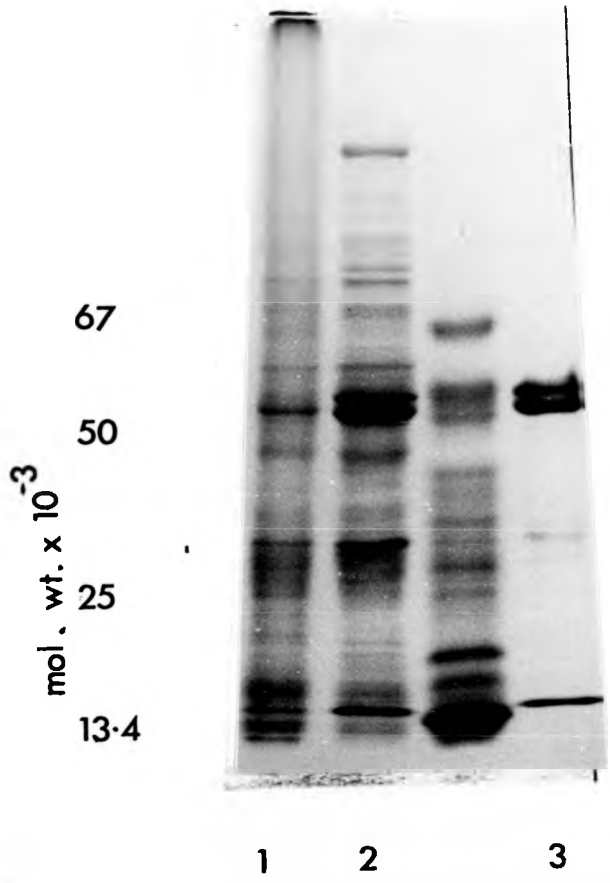


Fig. 26 SDS electrophoresis (7.5 to 20% w/v exponential gradient acrylamide gel) of various preparations obtained during purification of R. vannielii (RM5) RuBP carboxylase

(1) after $(NH_4)_2SO_4$ treatment (200 µg protein)
(2) after Ultrogel AcA22 chromatography (200 µg protein)
(3) after sedimentation into 0.2 to 0.8 M sucrose gradient (45 µg protein)

pure protein and enzyme solution at stages during purification were examined with this gel system (Fig. 26). Improved resolution of the two large subunit species was obtained. This heterogeneity was also evident following gel filtration on Ultrogel AcA 22. However, although a lower protein loading was used, the 56,200 mol.wt. subunit was not apparent after $(NH_4)_2\ SO_4$ precipitation suggesting that the heterogeneity was a consequence of the purification procedure. The quaternary structure, as revealed after a single purification step by sucrose density gradient centrifugation (see page 154), was not sufficiently resolved to confirm the nature and cause of the heterogeneity. However, the spinach RuBP carboxylase purified in a single step (see page 157), also apparently contained heterogeneous large subunits (Fig.32b). Whether these results with the spinach and R. vannielii (RM5) RuBP carboxylases arise as a result of protein aggregation, the purification buffer, PAGE system, etc., or are due to the intriguing suggestion that gene duplication and mutation has occurred (Purohit and McFadden, 1976), awaits further investigation.

Densimetric scans of a number of stained gels gave an average mole ratio of small to total large subunits of 1.15. With a molecular weight of 430,000, the most likely quaternary structure of this enzyme consists of six large and six small subunits. The only published report of an RuBP carboxylase

with a similar structure, is that of McFadden (1977) working with P. oxalaticus.

Purification of the R. vannielii (RM5) RuBP carboxylase from fresh cell-free extracts, clearly indicated the presence of both large and small polypeptide subunits. However, following storage of partially purified enzyme (following 35 to 60% (w/v) $(NH_4)_2$ SO_4 precipitation) for one month at 4^oC and to a lesser extent, -20^oC, prior to further purification, SDS-PAGE failed to reveal any small polypeptide subunits (Fig. 27). This indicates the susceptibility of the small subunit of R. vannielii (RM5) RuBP carboxylase to proteolysis or other forms of degradation. As indicated in the General Introduction, similar loss of the small subunit has been reported for the RuBP carboxylase of Aphanocapsa 6308 (Codd and Stewart, 1977) and E. halophila (McFadden and Tabita, 1974). Care must therefore be taken in determining the quaternary structure and it is suggested that during purification, harsh treatments (e.g. acid precipitation) be avoided. In view of the fact that most RuBP carboxylases so far characterised as lacking small subunits, have required lengthy purification procedures, a re-evaluation of their quaternary structure may be indicated. The development of rapid purification procedures (e.g. sucrose gradient centrifugation in fixed angle rotors; affinity chromatography) may be advantageous in this respect.

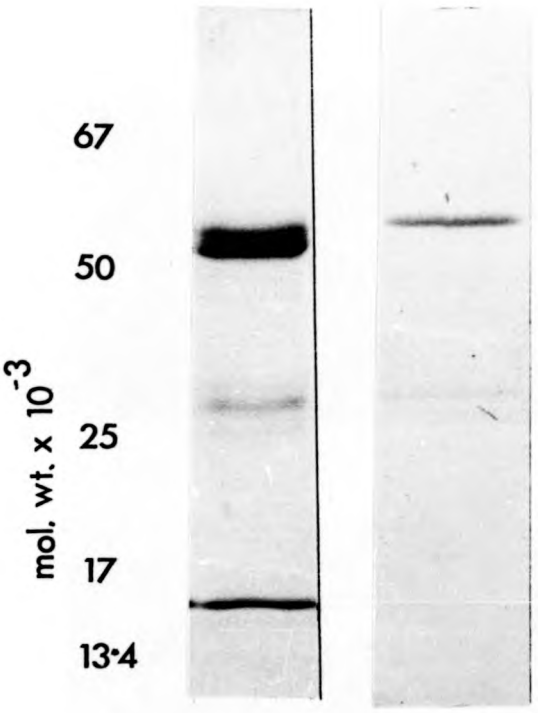
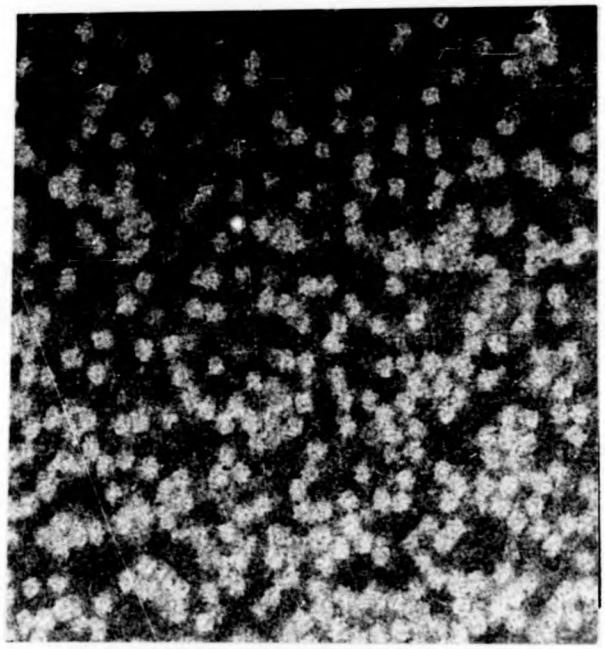


Fig. 27 SDS electrophoresis (7.5 to 20% w/v exponential gradient acrylamide gel) of (1) R. vannielii (RM5) RuBP carboxylase and (2) enzyme purified following storage after $(NH_4)_2SO_4$ treatment for 1 month at 4^oC



magnification x 250,000

Fig. 28 Electron micrograph of negatively stained R. vannielii (RM5) RuBP carboxylase

The overall structure of the RuBP carboxylase was examined by electron microscopy using negatively stained pure enzyme, as described in the Materials and Methods section (subsection 6) (Fig. 28). Although multisubunit structures were evident, magnification and resolution were insufficient for an evaluation of the three dimensional structure of the enzyme. It was not therefore possible to compare the R. vannielii (RM5) RuBP carboxylase containing six large and six small subunits, with the RuBP carboxylase of I. intermedius examined by Purohit et al. (1976).

h) CONCLUDING REMARKS ON THE NATURE OF THE R. VANNIELII (RM5) RuBP CARBOXYLASE

In conclusion, the RuBP carboxylase of R. vannielii (RM5) has properties similar to those of the large molecular weight class of RuBP carboxylases although a molecular weight of 430,000 is somewhat less than that assigned to this class.

Only one form of the enzyme was present in R. vannielii (RM5) unlike the situation reported in R. spheroides (Gibson and Tabita, 1977a) and R. capsulata (Gibson and Tabita, 1977b). The high molecular weight and the presence of small subunits, indicate that the R. vannielii (RM5) RuBP carboxylase is evolutionarily more advanced than the enzyme from R. rubrum

and the low molecular weight enzyme from R. spheroides and R. capsulata. This is in basic agreement with morphogenetic evolution as indicated by cell-cycle complexity (Fig. 12). The RuBP carboxylase of R. vannielii (RM5) will be further considered in the context of the evolution of autotrophy in section IV (subsection 11). The apparent instability of the R. vannielii (RM5) RuBP carboxylase quaternary structure has also been indicated and this fact should be borne in mind when investigating RuBP carboxylase structure.

7. ENZYME PURIFICATION BY SUCROSE DENSITY GRADIENT VELOCITY CENTRIFUGATION

a) Introduction

Goldthwaite and Bogorad (1971), first reported the use of sucrose density gradient velocity centrifugation as a means of purifying RuBP carboxylase from leaf tissue. This technique had several advantages over the conventional methods of RuBP carboxylase purification. It was relatively quick and also allowed complete recovery of protein. Furthermore, several different samples could be studied concurrently. The purification technique relied on the unusually high sedimentation coefficient and molecular weight of plant RuBP carboxylase compared to those of the bulk proteins. In plants, where the carboxylase may represent up

to 50% (w/w) of the total soluble protein, fractions from sucrose gradients with RuBP carboxylase activity yielded homogeneous enzyme in one step.

At the same time as the report of Goldthwaite and Bogorad (1971), Romanova et al. (1971) indicated that sucrose density gradient velocity centrifugation could be used in conjunction with $(NH_4)_2SO_4$ precipitation, to purify the RuBP carboxylase of A. eutropha Z-1. Tabita and McFadden (1974c) similarly used sucrose density gradient velocity centrifugation as a one-step technique to purify, or partially purify, a number of high molecular weight bacterial RuBP carboxylases. These procedures all involved the use of swinging bucket rotors and consequently centrifugation times of up to 24 h. Considerable loss of activity however, can occur, even with this one-step purification (G. A. Codd, University of Dundee, personal communication).

Sucrose gradients have also been extensively used for equilibrium centrifugation. Flamm et al. (1966) first indicated the advantages of using fixed angle rotors as opposed to swinging bucket rotors for equilibrium centrifugation, in the separation of nucleic acids and mixtures thereof. Greater resolution and increased loading capacity were found for gradients in fixed angle rotors. Johnson et al. (1973) extended this technique to compare fixed angle and

swinging bucket rotors for the separation of density labelled proteins from unlabelled proteins by equilibrium centrifugation. As with nucleic acids, fixed angle rotors offered greater resolution and shorter equilibration times than swinging bucket rotors. For both proteins and nucleic acids therefore, the fixed angle rotor has been the choice for equilibrium centrifugation.

Sucrose density gradient velocity centrifugation in fixed angle rotors has recently been used (S. N. Covey, University of Warwick, manuscript in preparation) for the purification of plant messenger ribonucleic acids (RNA) where instability necessitates their rapid purification. Separation of RNA has been achieved with centrifugation times of as little as one hour giving superior resolution to that found using a swinging bucket rotor with centrifugation times of over ten hours.

In view of the unstable nature of the R. vannielii (RM5) RuBP carboxylase, it was decided to investigate the possible use of sucrose density gradient velocity centrifugation in fixed angle rotors as a means of rapidly purifying RuBP carboxylases.

b) Purification of Rhodomicrobium vannielii (RM5) RuBP carboxylase by sucrose density gradient centrifugation in a swinging-bucket rotor

A partial purification of R. vannielii (RM5) RuBP carboxylase was achieved in a single step from crude cell-free extracts, by sedimentation into a four-step, 0.2 to 0.8M discontinuous sucrose gradient. Centrifugation was for 22 h in a 3 x 70 swinging bucket rotor and the profile of a typical gradient is shown in Figure 29. The peak of RuBP carboxylase activity ran slightly in front of the bulk proteins and pigmented material, the majority of which remained at the top of the gradient. The specific activity of the combined fractions containing RuBP carboxylase activity was 0.6 units.mg.protein^{-1} representing a 27-fold purification from crude cell-free extract. The failure of RuBP carboxylase to be completely separated from the bulk proteins, presumably indicates a lower sedimentation coefficient than those of the RuBP carboxylases investigated by Tabita and McFadden (1974c). This is reflected in the lower molecular weight (430,000) of the R. vannielii (RM5) enzyme. A relatively broad peak of activity for the carboxylase was obtained, probably due to diffusion of the protein over the long centrifugation time. Fairly broad peaks were also obtained by Tabita and McFadden (1974c), Goldthwaite and Bogorad (1971), and other workers using this technique.

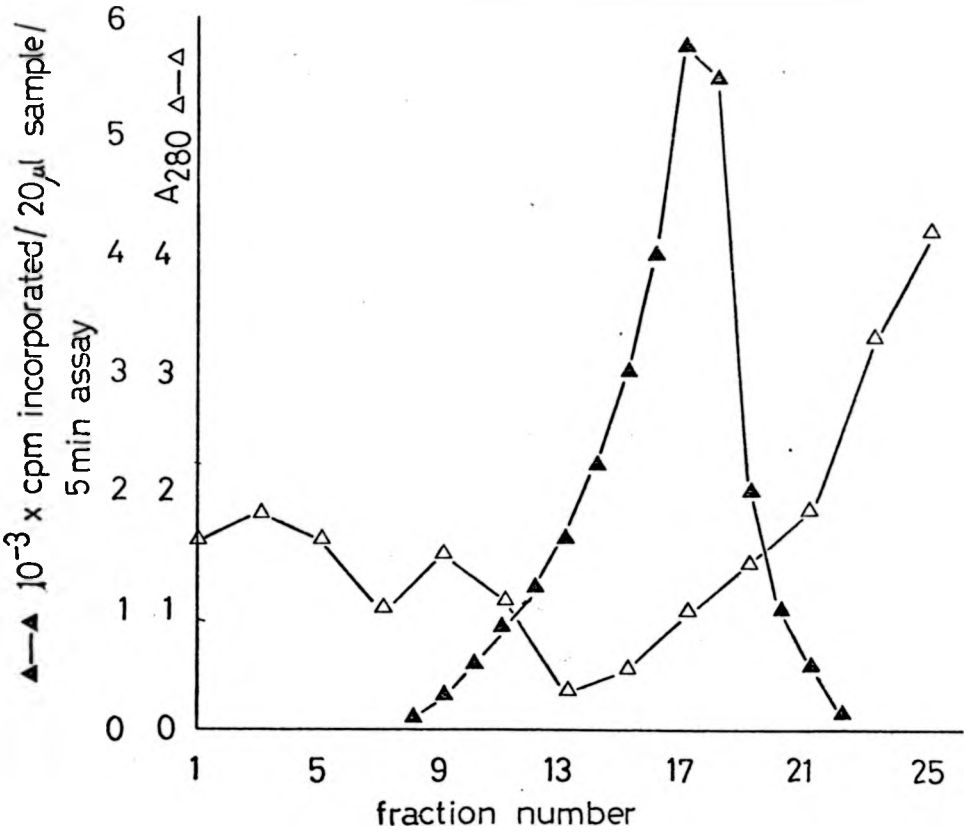


Fig. 29 Sucrose density gradient centrifugation of R. vannielii (RM5) RuBP carboxylase

25 g of protein as a cell-free extract was applied to the gradient which was centrifuged for 22 h in a 3 x 70 swinging bucket rotor (see Materials and Methods section)

The choice of which swinging bucket rotor is to be used, obviously influences the resolution that is possible and the centrifugation time required. A balance has to be drawn between a long, thin centrifuge tube, giving the optimum resolution and shortest centrifugation time, but on which only small quantities of protein can be loaded, and a shorter, wider centrifuge tube on which larger quantities of protein can be loaded but which gives poorer resolution and longer centrifugation times. For preparative work, a rotor taking tubes of at least 40 ml capacity is generally used. The need to draw this balance is not so evident with fixed angle rotors as will be indicated below.

c) The use of fixed angle rotors for RuBP carboxylase purification

Figure 30 a,b,c represents the fractionation of linear 0.2 to 0.8M sucrose gradients centrifuged in a fixed angle rotor, as described in the Materials and Methods section (subsection 22) for 1 h, 1 h 20 min and 1 h 40 min respectively. 25 mg of protein (0.8 ml) as a crude cell-free extract of R. vannielii (RM5), was loaded onto each gradient. Following centrifugation for 1 h the RuBP carboxylase peak was poorly separated from bulk proteins at the top of the gradient. This is indicated by a specific activity of the pooled active fractions of 0.45 units.mg.protein^{-1}. After centrifugation for 1 h 20 min

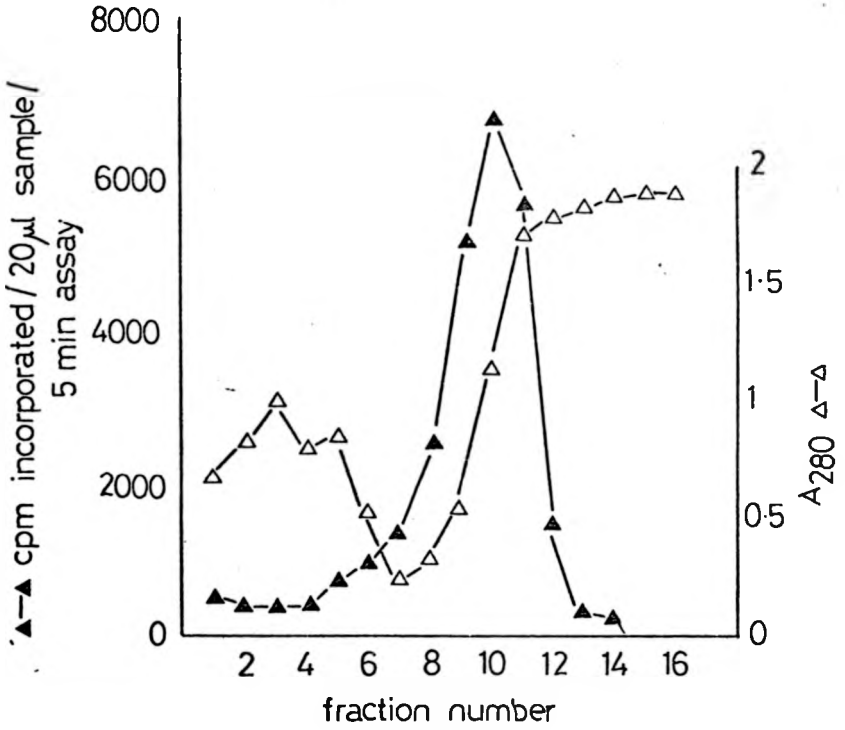


Fig. 30a Sucrose density gradient centrifugation of R. vannielii (RM5) RuBP carboxylase

25 mg of protein as a cell-free extract was applied to the gradient which was centrifuged for 1 h in an 8 x 25 fixed angle rotor (see Materials and Methods section)

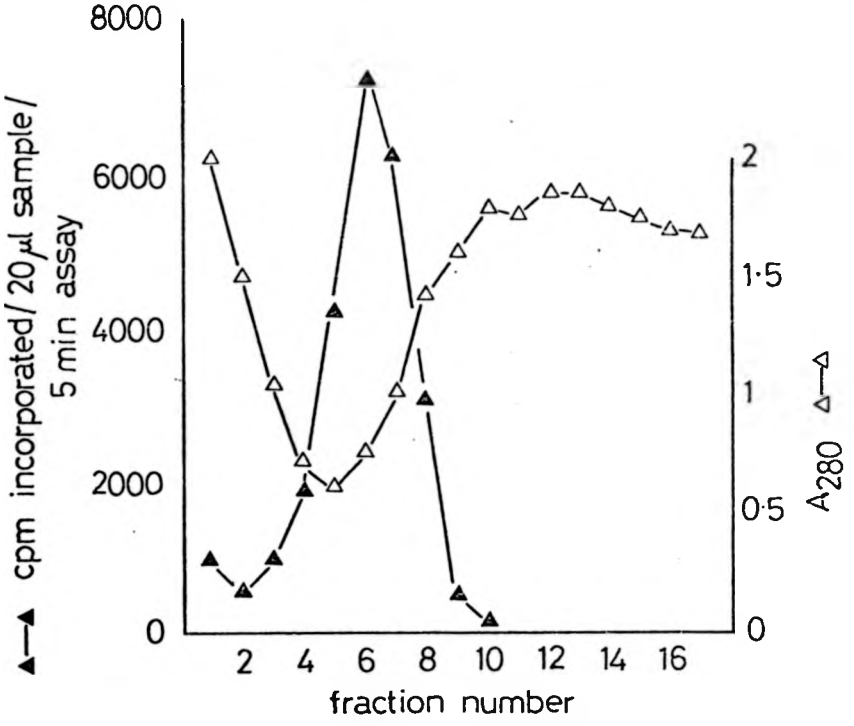


Fig. 30b Sucrose density gradient centrifugation of R. vannielii (RM5) RuBP carboxylase

25 mg of protein as a cell-free extract was applied to the gradient which was centrifuged for 1 h 20 min in an 8 x 25 fixed angle rotor (see Materials and Methods section)

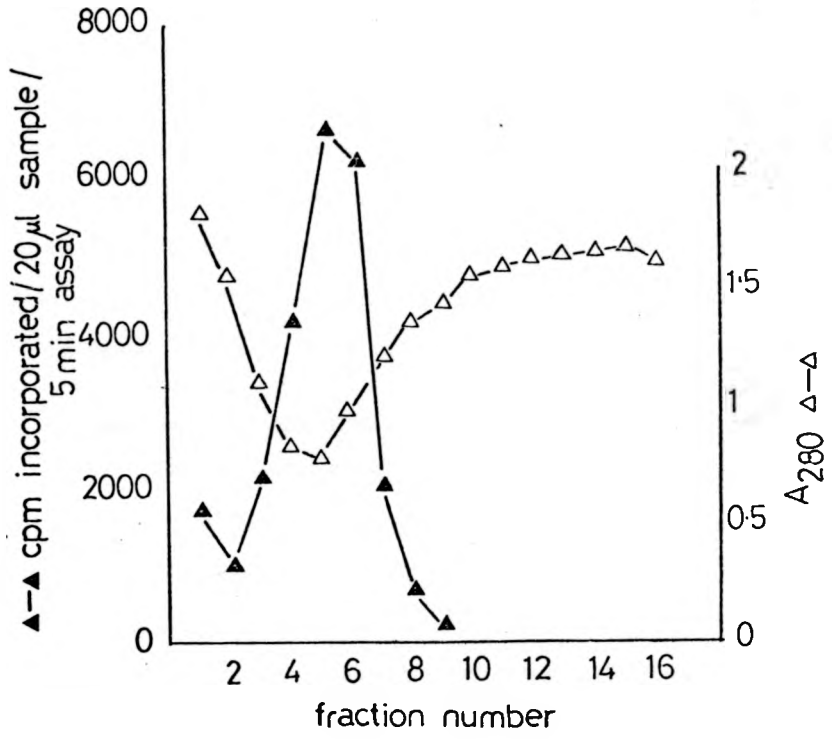


Fig. 30c Sucrose density gradient centrifugation of R. vannielii (RM5) RuBP carboxylase

25 mg of protein as a cell-free extract was applied to the gradient which was centrifuged for 1 h 40 min in an 8 x 25 fixed angle rotor (see Materials and Methods section)

and 1 h 40 min, the enzyme had sedimented further down the gradient with improved separation from the bulk proteins. Specific activities of pooled active fractions were 1.1 units. $mg.protein^{-1}$ and 0.88 $units.mg.protein^{-1}$ respectively, representing at maximum, an approximate 50-fold purification from crude cell-free extract with an initial specific activity of 22 $munit.mg.protein^{-1}$.

Peaks of enzyme activity were not symmetrical but slightly skewed toward the leading edge of the sedimenting protein. This is probably due to the technique for fractionating the gradient (S. N. Covey, personal communication) or to sedimenting molecules hitting the wall of the centrifuge tube, an effect not present with the swinging bucket rotor. However, any 'wall effects' did not lead to significant pelleting of RuBP carboxylase. Furthermore, peaks of activity were found to be less broad than with gradients from swinging bucket rotors, presumably due to less diffusion of protein.

The distribution of proteins within the sucrose gradient after centrifugation of R. vannielii (RM5) cell-free extract for 1 h 20 min, was examined by SDS-PAGE. Dissociated proteins from each fraction were separated on a 7.5 to 20% (w/v) exponential gradient, polyacrylamide gel (Fig. 31). The location of the majority of proteins at the top of the gradient is clearly indicated. Fractions 6 and 7 contained the maximum

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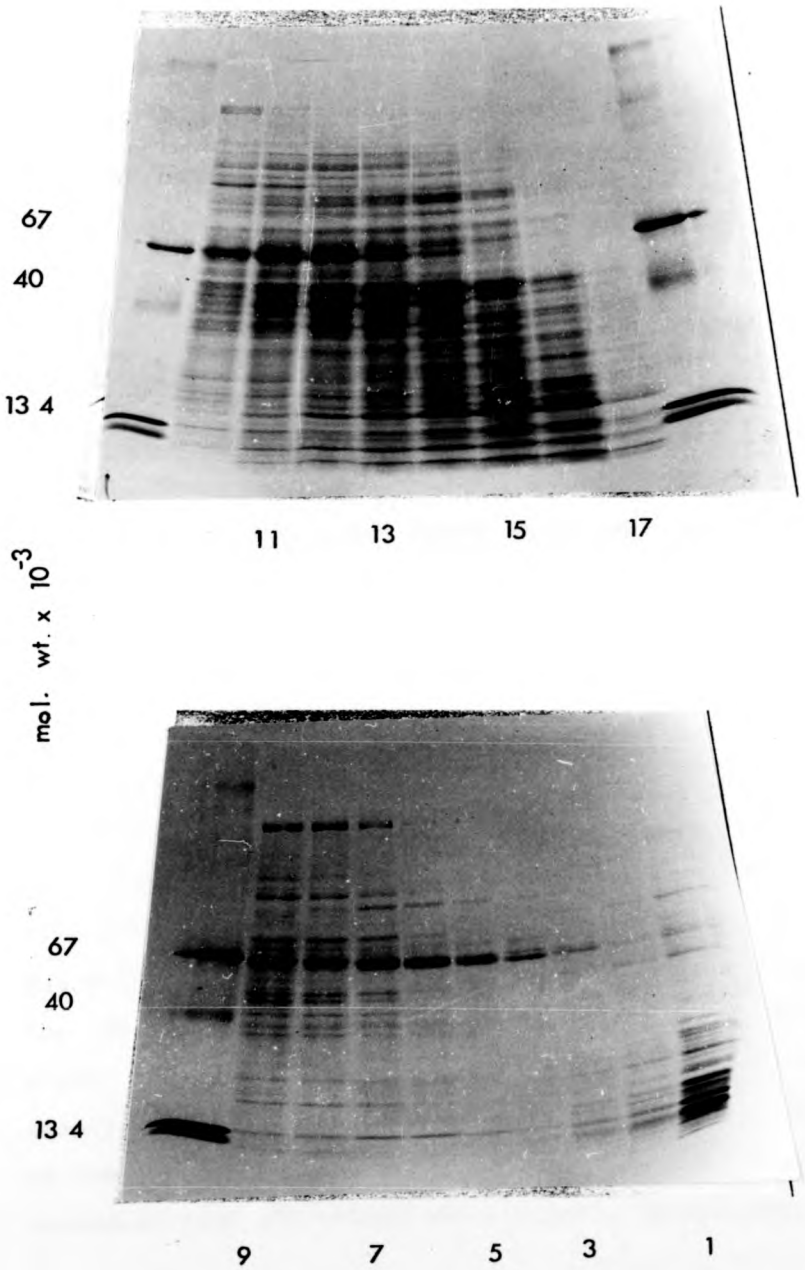
Fig. 31 SDS electrophoresis (7.5 to 20% w/v exponential gradient acrylamide gels) of fractions from a sucrose density gradient centrifuged for 1 h 20 min in an 8 x 25 fixed angle rotor (see Fig. 30b) 25 mg R. vannielii (RM5) cell-free extract was applied to the gradient. 100 µl of each fraction used per gel track. Fraction 1 corresponds to the base of the gradient (0.8 M sucrose)


67
40
13 4
11
13
15
17
mol. wt. x 10^{-3}
67
40
13 4
9
7
5
3
1

RuBP carboxylase activity and stained bands, corresponding to large and small polypeptide subunits, peak in intensity in these fractions. As with gradients in the swinging bucket rotor, RuBP carboxylase was not homogeneous with a number of contaminating proteins present.

The separation of the R. vannielii (RM5) RuBP carboxylase from bulk proteins was improved by loading lower amounts of protein on each gradient. The 25 mg.protein per gradient loading used in the above experiments, was considerably higher than the quantity of protein used for each gradient in the purification of plant (Goldthwaite and Bogorad, 1971) and bacterial (Tabita and McFadden, 1974c) RuBP carboxylases. The use of lower protein loads was then investigated and the distribution of both R. vannielii (RM5) and spinach soluble proteins, in gradients centrifuged for 1 h 20 min in a fixed angle rotor examined (Fig. 32 a,b). In each case 5 mg.protein (0.5 ml solution) was loaded onto the gradient. RuBP carboxylase was well separated from bulk proteins which again remained at the top of the gradient. Spinach RuBP carboxylase had a higher rate of sedimentation than the R. vannielii (RM5) enzyme, consistent with its higher molecular weight (550,000). SDS-PAGE of the combined peak fractions of spinach RuBP carboxylase indicated that the enzyme was virtually homogeneous (Fig. 33).

Fig. 32a Sucrose density gradient centrifugation of spinach RuBP carboxylase

5 mg of protein as a cell-free extract was applied to the gradient which was centrifuged for 1 h 20 min in an 8 x 25 fixed angle rotor

Fig. 32b Sucrose density gradient centrifugation of R. vannielii (RM5) RuBP carboxylase

5 mg of protein as a cell-free extract was applied to the gradient which was centrifuged for 1 h 20 min in an 8 x 25 fixed angle rotor

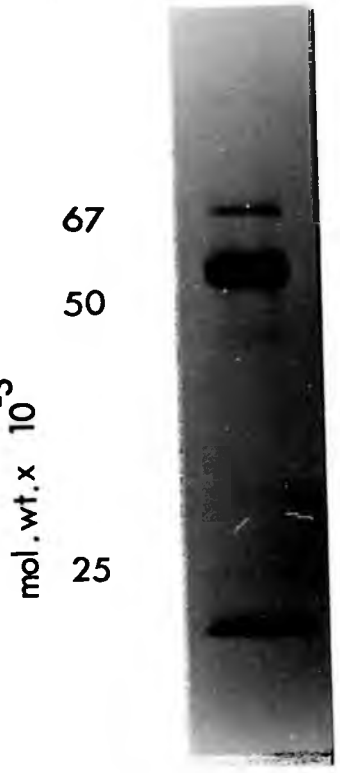


Fig. 33 SDS-electrophoresis (7.5 to 20% w/v exponential gradient acrylamide gel) of spinach RuBP carboxylase purified by sucrose density gradient centrifugation in a fixed angle rotor (see Fig. 32b)

The above experiments have shown that the resolution of proteins is dependent upon centrifugation time. This was further demonstrated by examining the distribution of a range of protein standards within sucrose gradients centrifuged in a fixed angle rotor, (Fig. 34 a,b). Separation of glutamate dehydrogenase, catalase and alcohol dehydrogenase was very much improved after centrifugation for 1 h 30 min than for 1 h 15 min. The distribution of these proteins within the gradient also corresponded to their respective molecular weights and sedimentation coefficients. However, it is not proposed that this technique be used for anything other than qualitative work.

The improved resolution and decreased centrifugation times found with the fixed angle rotor in practice, may be predicted theoretically from a consideration of the gradient during centrifugation. A scale diagram of a centrifuge tube in the 8 x 25 titanium angle rotor is shown in Figure 35 a,b. The sucrose gradient is represented as a series of bands. In the inclined orientation (Fig.35 a), the gradient is contracted to only 0.61 of the tube length and there is a corresponding increase in surface area of the liquid/air interface. Molecules therefore sediment as a much narrower band and have less distance to travel to reach an equivalent position in the gradient than would be the case with the swinging bucket rotor, where the gradient is effectively the

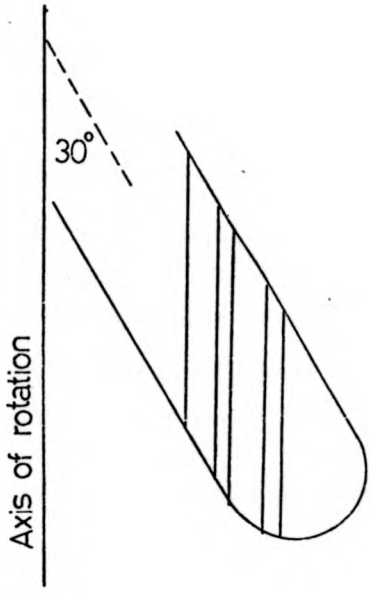
Fig. 34 Sucrose density gradient centrifugation of proteins in a fixed angle rotor

glutamate dehydrogenase, mol.wt. 320,000;
catalase, mol.wt. 232,000;
alcohol dehydrogenase, mol.wt. 141,000.

a) centrifugation for 1 h 15 min

b) centrifugation for 1 h 30 min

(a)



(b)

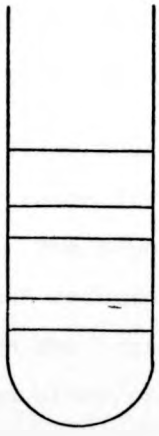


Fig. 35 Scale diagram of sucrose gradients in inclined (a) and collecting (b) positions for use in an 8 x 25 titanium angle rotor

same as when the tube is in its collecting position (Fig. 35 b) The increase in surface area of the liquid/air interface allows for a greater amount of sample to be loaded on each gradient. Equivalent results from a swinging bucket rotor would require a larger gradient and consequently both a longer centrifugation time and greater sample dilution. The mathematical derivation of these effects has been discussed by Flamm et al. (1966).

Two problems that one would predict may be of importance when using fixed angle rotors, are the re-alignment of the gradient when moving from inclined (Fig. 35a) to collecting (Fig. 35b) positions and the possible effects of sedimenting molecules hitting the wall of the tube. In practice, these do not appear to have significantly reduced resolution.

In conclusion, the fixed angle rotor has several advantages over the swinging bucket rotor for sucrose density gradient, velocity centrifugation. The high loading capacity per gradient and the very much reduced centrifugation times have obvious advantages. With the instability of many RuBP carboxylases and numerous other proteins, the ability to obtain pure enzyme from whole cells, in a matter of a few hours, may be of great importance.

Undescribed preliminary experiments have indicated that aluminium fixed angle rotors may be used in the absence of a titanium rotor, although centrifugation time needs to be increased to meet the lower centrifugation speed that has to be employed. The use of a vertical centrifuge rotor would no doubt improve resolution and loading capacity even further and alleviate any problem from 'wall effects'.

8. PARTIAL PURIFICATION OF RHODOSPIRILLUM RUBRUM RuBP CARBOXYLASE

The finding that the small subunits of R. vannielii (RM5) RuBP carboxylase were not always detectable, casts doubt on the reports of RuBP carboxylases characterised as comprising only large subunits. The presence or absence of small subunits has been a major characteristic used in describing the possible evolution of autotrophy (McFadden and Tabita, 1974; McFadden, 1975) with the enzyme from R. rubrum being considered the most primitive. In view of this, it was decided that the quaternary structure of the R. rubrum RuBP carboxylase warranted further investigation.

It was initially hoped to effect a partial purification in one step from crude cell-free extract by sucrose density

gradient centrifugation in a fixed angle rotor and then to examine each fraction of the gradient by SDS-PAGE to locate dissociated proteins occurring in maximum quantity where RuBP carboxylase activity was at a maximum. This procedure would very much reduce purification time and lessen the chance of possible subunit loss by degradation. However, the low sedimentation coefficient and molecular weight (114,000) of the R. rubrum RuBP carboxylase, did not allow for sufficient separation from bulk proteins. Consequently, a DEAE cellulose chromatography step was used, prior to sucrose gradient centrifugation, to remove part of the contaminating protein. The protocol for this partial purification is shown in Table 7.

As with the R. vannielii (RM5) enzyme, R. rubrum RuBP carboxylase eluted from the DEAE column as a single peak of activity indicating the presence of only one form of the enzyme (Fig. 36). Following sucrose density gradient centrifugation, the enzyme remained close to the top of the gradient with the bulk proteins. SDS-PAGE of each fraction across the activity peak indicated the presence of considerable contaminating protein (Fig. 37). However, a stained band corresponding to a molecular weight of approximately 50,000 did show maximum intensity in the fraction with maximum enzyme activity, presumably the large subunit. Although obviously not conclusive, no stained band corresponding to a small subunit was observed.

Table 7 Partial purification of RuBP carboxylase from photoheterotrophically grown R. rubrum

STEP	SPECIFIC ACTIVITY (units.mg.protein^{-1})	PURIFICATION FACTOR
100,000 g for 1 h supernatant	0.010	-
DEAE chromatography (pooled & concentrated active fractions)	0.056	5.6
0.2 to 0.8 M sucrose gradient (peak active fraction)	0.143	14.3

1 unit = 1 μmol CO_2 fixed.min^{-1}

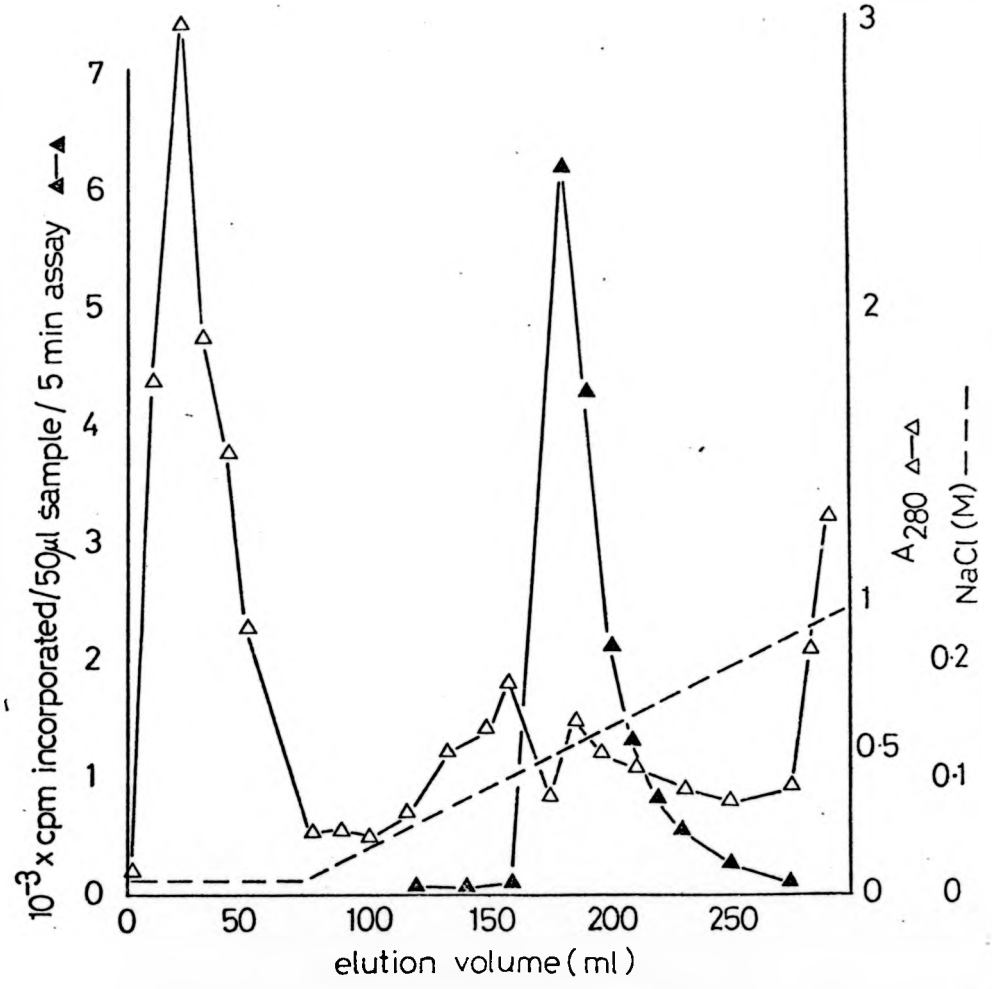


Fig. 36 DEAE cellulose chromatography of R. rubrum RuBP carboxylase

Fig. 37 Sucrose density gradient centrifugation of *R. rubrum* RuBP carboxylase

Gradient centrifuged for 1 h 20 min in an 8 x 25 fixed angle rotor

100 µl samples from fractions of the gradient across the RuBP carboxylase activity peak were examined by SDS electrophoresis (7.5 to 20% w/v exponential gradient acrylamide gel)

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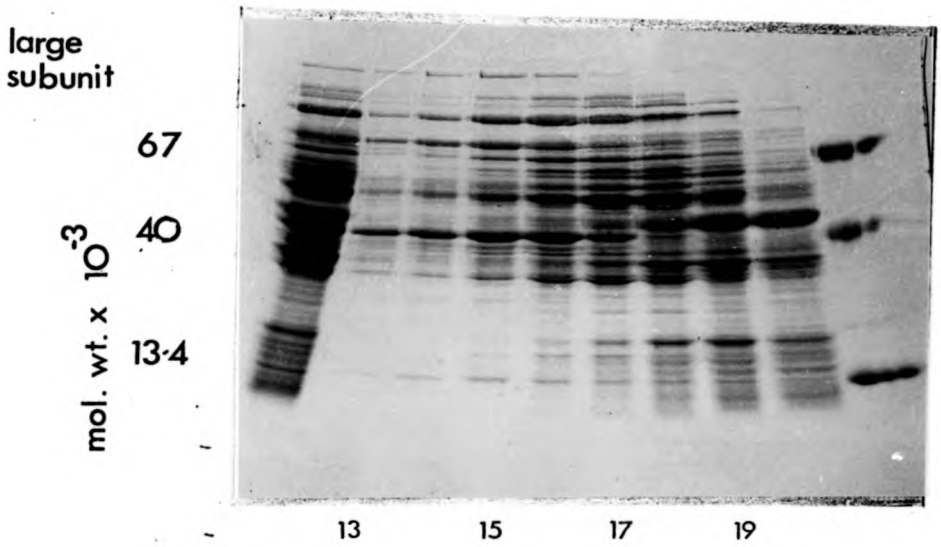

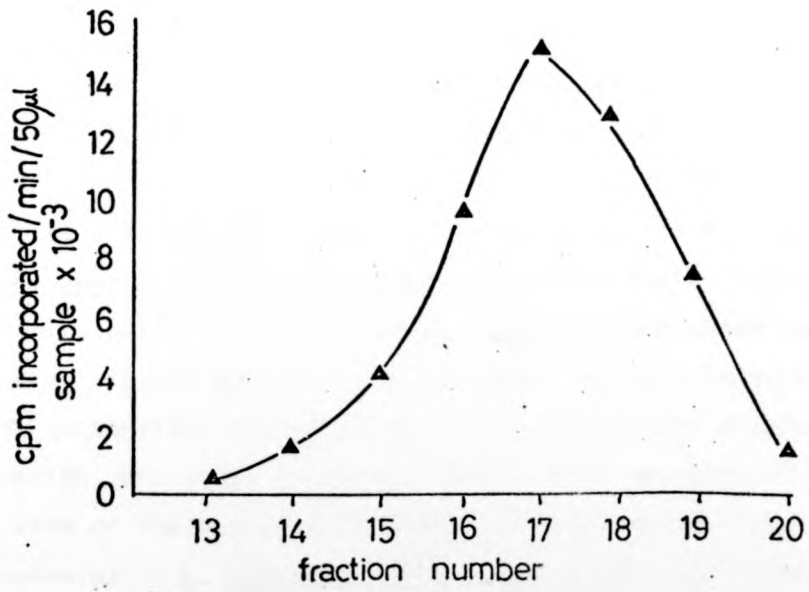

This result is in agreement with the work of Tabita and McFadden (1974b). However, the possibility that small subunits are lost from the enzyme on cell lysis remains.

9. IN VIVO STUDIES ON CARBON DIOXIDE FIXATION AND RuBP CARBOXYLASE ACTIVITY IN RHODOMICROBIUM VANNIELII (RM5)

The majority of the work on the regulation of carbon dioxide assimilation and RuBP carboxylase has been done using R. rubrum, with particularly interesting studies by Slater and Morris (1973a, b). The level of RuBP carboxylase in cell-free extracts of R. vannielii (RM5) as a response to carbon growth substrate, has already been shown in this thesis, to differ from the reported results for R. rubrum (Tabita and McFadden, 1974a). Furthermore, the RuBP carboxylase of R. vannielii (RM5) was shown to contain small 'regulatory' subunits unlike the enzyme of R. rubrum. With these points in mind, it was decided to investigate the relationship between RuBP carboxylase activity and the in vivo carbon dioxide fixation rate of R. vannielii (RM5). This was also of interest in view of the reported occurance of 'carboxysome-like' structures in R. vannielii (RM5) under conditions of high carbon dioxide tension and low growth rate (France, 1978).

a) The changing rates of carbon dioxide fixation and enzyme activity during a batch growth cycle on malate medium

The rate of whole cell carbon dioxide fixation and the activity of RuBP carboxylase in cell-free extracts at various stages during a batch growth cycle of R. vannielii (RM5) on malate medium, is shown in Figure 38 a. The inoculum for this experiment was taken from a malate grown culture in the late exponential phase of growth. Carbon dioxide fixation had a maximum rate of 0.54 μmol.h^{-1}.(mg.dry wt)$^{-1}$, attained in the very early exponential phase. This was a $3\frac{1}{2}$-fold increase over the rate of carbon dioxide fixation by the inoculum. After the early exponential phase, the fixation rate fell to a value of 0.14 μmol.h^{-1}.(mg.dry wt)$^{-1}$, similar to that of the inoculum. The changing rate of carbon dioxide fixation was however, not paralleled by equivalent changes in RuBP carboxylase activity. This remained relatively constant throughout the growth cycle with a maximum specific activity of 48 munit.mg. protein^{-1}, seen in mid to late exponential phase cells. The activity of RuBP carboxylase in the particulate fraction of cell-free extracts remained negligible at all stages of the growth cycle. These results are clearly different to those reported for R. rubrum by Slater and Morris (1973a), where changes in RuBP carboxylase activity and carbon dioxide fixation rate paralleled each other. It may be implied from the results with R. vannielii (RM5), that there is no significant change

Fig. 38a Changes in the activity of RuBP carboxylase and rate of whole cell carbon dioxide fixation during a batch growth cycle of R. vannielii (RM5) on malate medium

Fig. 38b Changes in the activity of PEP carboxylase and phosphoribulokinase during a batch growth cycle of R. vannielii (RM5) on malate medium

in the rate of synthesis of RuBP carboxylase during the batch growth cycle which may suggest that in R. vannielii (RM5), the enzyme is constitutive. Consequently, if regulation of carbon dioxide fixation is effected by RuBP carboxylase, as it would appear to be in R. rubrum, then it is brought about by changes in the degree of inhibition of activity rather than by altered rates of synthesis.

However, it would seem more likely that carbon dioxide fixation is controlled at another enzyme location, possibly not directly involved with the Calvin cycle. Likely candidates would be phosphoribulokinase or other carboxylase enzymes present in R. vannielii (RM5) and the activities of these enzymes are shown in Figure 38b. As with RuBP carboxylase, changes in activity did not parallel changes in carbon dioxide fixation rate, and it is interesting that the specific activities of the C_3-carboxylases were considerably less than that of the RuBP carboxylase.

Obviously, these results are far from conclusive and require further work in order to be substantiated. However, in view of the close biochemical relationship between R. rubrum and R. vannielii (RM5), it is tempting to suggest that the differing response in RuBP carboxylase activity is in some way due to the presence of small subunits in the R. vannielii (RM5) enzyme. It is possible that the enzyme can exist in both

active and inactive forms within the cell and that on cell-lysis, all enzyme is converted to an active form. On the basis of this hypothesis, it was thought that an inactive form of the enzyme may be present as carboxysomes, which would indeed break down on cell-lysis. However, no evidence could be found to support this in that only negligible RuBP carboxylase activity was associated with the particulate fraction of cell-free extracts and also ultra-thin sections of cells made at stages during the growth cycle failed to reveal 'carboxysome-like' structures when viewed by electron microscopy.

It would be interesting to extend these studies to cover a range of organisms possessing a Calvin cycle, to see if there is a correlation between carbon dioxide fixation and RuBP carboxylase activity, and the presence or absence of small subunits in their respective RuBP carboxylases. Furthermore, with the ability to purify RuBP carboxylase quantitatively in one step from crude cell extracts, by sucrose density gradient centrifugation, it would be of interest to measure the absolute amount of enzyme present at stages through the growth cycle to see if the measured changes in activity are a true reflection of enzyme synthesis.

b) The effect of dilution rate on the activity of RuBP carboxylase during chemostat continuous culture on PM-medium

To further investigate the control of RuBP carboxylase synthesis, enzyme activity in cell-free extracts was measured at a series of dilution rates in a chemostat, with carbon limitation. The results are shown in Table 8 Little gross change occurred in activity of the RuBP carboxylase with changing dilution rate although activity was noticeably higher at the lower dilution rates, similar to the results of Slater and Morris (1973a) with R. rubrum. However, the low concentration of carbon that was presumably present at low growth rates (cf Herbert et al., 1956), did not result in any major increase in RuBP carboxylase activity as suggested by Knight et al. (1978). This may then suggest that RuBP carboxylase synthesis is not repressed in R. vannielii (RM5) by the presence of a heterotrophic substrate or a metabolic derivative thereof. This hypothesis is consistent with the high level of RuBP carboxylase in cell-free extracts prepared following growth on malate, relative to that following autotrophic growth.

The possible occurrence of carboxysomes at low dilution rates was investigated by the examination of ultra-thin sections of cells by electron microscopy. At a dilution rate

Table 8 The effect of dilution rate on the activity of RuBP carboxyla e cell-free extracts of *R. vannielii* (RM5) grown in chemostat continuous culture as described in Materials and Methods section

Dilution rate (h^{-1})	RuBP carboxylase activity (munit.mg protein^{-1})
0.015	27
0.026	24
0.035	19
0.048	20
0.100	10

of 0.026 the carbon dioxide tension in the chemostat vessel at steady state, varied between 8 and 10.5% (v/v), similar conditions to those indicated by France (1978). However, it was not possible to repeat the observation of carboxysome-like structures in R. vannielii (RM5).

It must be emphasised that the results presented above for R. vannielii (RM5) are only preliminary and considerably more research must be done before any conclusive statements can be made. It does appear however, that synthesis of RuBP carboxylase is not of great importance as a mechanism for regulation of carbon dioxide fixation in this organism. Results as they stand do suggest a different mode of regulation to that observed in R. rubrum and further studies on these and other carbon dioxide assimilating autotrophs will no doubt yield some interesting observations.

SECTION IV

RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE AND CARBON DIOXIDE FIXATION IN *METHYLOCOCCUS CAPSULATUS* (BATH)

1. INTRODUCTION

The close physiological and morphological relationship that exists between the methylotroph, M. capsulatus and the nitrifying bacteria, organisms which derive the bulk of their cellular carbon from carbon dioxide, has been discussed in the General Introduction and more extensively, by Whittenbury and Kelly (1977). Enzyme studies and radiotracer work have indicated an unusual pattern of carbon metabolism in M. capsulatus (Bath) such that both RMP and serine pathways of carbon assimilation, may be present (Reed, 1976). With these points in mind, it was decided to investigate the possibility that some carbon is also assimilated in this organism as carbon dioxide via a Calvin cycle.

2. EVIDENCE FOR THE PRESENCE OF RuBP CARBOXYLASE AND PHOSPHORIBULOKINASE IN METHYLOCOCCUS CAPSULATUS (BATH)

RuBP and its immediate precursors in the Calvin cycle, ribulose 5-phosphate (Ru5P) and ribose 5-phosphate (R5P), were tested for their ability to act as carboxylation substrates for enzymes present in the soluble fraction of cell-free extracts of M. capsulatus (Bath) grown as a batch

culture (100 l fermenter). Activation of carbon dioxide fixation by ATP and/or β-NADH, as reported in Nitrobacter winogradskyi (Kiesow et al., 1977), was also investigated (Table 9).

In the absence of any substrate, carbon dioxide fixation was negligible. Carbon dioxide fixing activity was however evident in the presence of RuBP with a specific activity of 9.4 munit.mg.protein^{-1}. This activity was lost following heat treatment of the extract as would be expected for an enzymically catalyzed reaction. Neither ATP nor β-NADH had any significant effect on the fixation rate. Ru5P and R5P both required ATP for activity suggesting that these substrates need to be converted to RuBP via a phosphoribulokinase before carbon dioxide fixation can occur. A phosphoriboisomerase, needed to interconvert Ru5P and R5P, has been previously reported in M. capsulatus (Kemp, 1972). With all three substrates, a decrease in carbon dioxide fixing activity was observed in the presence of β-NADH, the reason for which is at present unclear. This is however different to the stimulation of carbon dioxide fixation by β-NADH observed in R. vannielii (RM5) (section III.3). Comparatively low activities were observed for the carboxylase when using either Ru5P or R5P as test substrate. This suggests that the kinase, needed to convert the pentose monophosphates to the bisphosphate,

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Table 9 The fixation of carbon dioxide by cell-free soluble extracts of *Methylococcus capsulatus* (Bath)

Test substrate	CO_2 fixation (nmol CO_2 fixed.min^{-1} mg $protein^{-1}$)
None	0.2
Ribulose 1,5-P_2	9.4
Ribulose 1,5-P_2 (boiled extract)	0.3
Ribulose 1,5-P_2 + ATP	10.1
Ribulose 1,5-P_2 + NADH	9.0
Ribulose 1,5-P_2 + ATP + NADH	9.0
Ribulose 5-P	0.4
Ribulose 5-P + ATP	3.3
Ribulose 5-P + NADH	0.5
Ribulose 5-P + ATP + NADH	2.4
Ribose 5-P	0.2
Ribose 5-P + ATP	2.5
Ribose 5-P + NADH	0.3
Ribose 5-P + ATP + NADH	1.8

is either present in a low amount or else requires activation.

The possibility that phosphoribulokinase was partly associated with the particulate fraction of cell-free extracts was examined by measuring RuBP and R5P stimulated carbon dioxide fixation by crude cell-free extract comprising both particulate and soluble fractions (Table 10). A low activity for the carboxylase was still measured when R5P was used as test substrate, although this activity was greater than when using only the soluble fraction of cell-free extract. Addition of β-NADH again resulted in a concentration dependent decrease in activity.

The specific activity of carbon dioxide fixation, although low in comparison to that observed in extracts of autotrophs grown with carbon dioxide as their sole source of carbon, is comparable to that found in extracts of heterotrophically grown R. rubrum (see section III.8 and Slater and Morris, 1973a) and Chlorobium thiosulfatophilum (Tabita et al., 1974). In this context it should be realized that Methylococcus derives most of its carbon from methane without the intermediate formation of carbon dioxide, and consequently, carbon dioxide fixation only represents a small proportion of the total carbon assimilated.

Table 10 The fixation of carbon dioxide by crude cell-free extracts of Methylococcus capsulatus (Bath) (particulate and soluble fractions)

Test substrate	CO_2 fixation nmol CO_2 fixed.min^{-1}.mg $protein^{-1}$
None	0.1
Ribulose 1,5-P_2	13.9
Ribulose 1,5-P_2 + ATP	14.1
Ribulose 1,5-P_2 + NADH	13.6
Ribulose 1,5-P_2 + ATP + NADH	13.7
Ribose 5-P	0.3
Ribose 5-P + ATP	6.0
Ribose 5-P + 0.4mM NADH	2.2
Ribose 5-P + 0.8mM NADH	1.8
Ribose 5-P + 1.2mM NADH	1.2

The RuBP carboxylase activity in M. capsulatus (Bath) was further characterized by analysing the products formed in the cell-free, carbon dioxide fixation assay by two dimensional chromatography, as described in the Materials and Methods section. With enzyme partially purified by sucrose gradient centrifugation (see this section, 4e), a single radioactive spot was detected (Fig. 39) which co-chromatographed with 3-phosphoglyceric acid. Using the soluble fraction of cell-free extract as source of enzyme, two spots were detected, one being 3-phosphoglycerate and the second less intense spot, phosphoenolpyruvate, presumably formed from 3-phosphoglycerate by phosphoglyceromutase and enolase present in the extract.

M. capsulatus (Bath) assimilates the bulk of its carbon from methane via 3-hexulose phosphate synthase. This enzyme catalyses the condensation of a one-carbon unit (formaldehyde) with a five-carbon acceptor (Ru5P; Ferenci et al., 1974) and a possible evolutionary link between this enzyme and RuBP carboxylase, has been discussed by McFadden (1975). To determine whether RuBP carboxylase activity in M. capsulatus (Bath) was mediated by this enzyme both soluble and particulate fractions of cell-free extract were assayed for 3-hexulose phosphate synthase activity (Table 11). However, this possible dual role of 3-hexulose

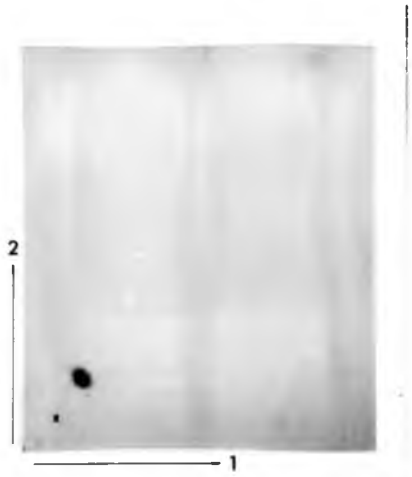

Fig. 39 Two dimensional chromatography of the assay products of partially purified M. capsulatus (Bath) RuBP carboxylase

Table 11 The activities of 3-hexulose phosphate synthase and RUBP carboxylase in soluble and particulate fractions from crude cell-free extracts of Methylococcus capsulatus (Bath)

Fraction	3-Hexulose phosphate synthase (nmol substrate fixed.min^{-1} mg protein^{-1})	Ribulose 1,5-bisphosphate carboxylase (nmol CO_2 fixed.min^{-1} mg protein^{-1})
Soluble	12	10.5
Particulate	306	2.9

phosphate synthase was discounted because the synthase activity was almost entirely associated with the particulate fraction in crude extracts, whereas the carboxylase activity was found in the soluble fraction.

This data presented above has demonstrated the presence of RuBP carboxylase and phosphoribulokinase in extracts of methane grown M. capsulatus (Bath).

3. DISTRIBUTION OF RuBP CARBOXYLASE AMONGST METHYLOTROPHS

Having found RuBP carboxylase in one methane-oxidiser, a survey of its distribucion in other strains was undertaken. Cell-free extracts of a number of 'type I' and 'type II' methylotrophs plus two trimethylamine utilisers, bacterium C2A1 and bacterium 4B6, were tested for RuBP carboxylase activity (Table 12). Significant enzyme activity was found only in extracts of the two Methylococcus species examined, with M. capsulatus (Bath) having the greater specific activity. The very low level of activity observed in M. methanica (S1) was not further investigated and its importance is at present unclear. However, the possibility of contamination was not rigorously ruled out and RuBP carboxylase has been previously reported as

Table 12 The specific activity of RuBP carboxylase in cell-free extracts of a range of methylotrophs

Organism	Specific activity RuBP carboxylase munit.mg protein^{-1}
Type 1 methylotrophs	
Methylomonas albus (BG8)	0
Methylomonas methanica (S1)	0.2
Methylomonas agile	0
Methylococcus capsulatus (Bath)	13.6
Methylococcus capsulatus (Foster & Davis)	4.1
Type 2 methylotrophs	
Methylosinus trichosporium (OB3b)	0
Methylocystis parvum (OBBP)	0
Methylosinus sporium	0
bacterium C2A1	0
bacterium 4B6	0

undetectable in M. methanica (Kemp and Quayle, 1966). Since the published report of RuBP carboxylase in M. capsulatus (Bath), the enzyme has been found in another Methylococcus strain (G. Codd, University of Dundee, personal communication). That there are problems associated with the classification of Methylococcus either as a type I or type II methane-oxidiser, has been indicated in the General Introduction. The presence of RuBP carboxylase further suggests that Methylococcus should belong to a third group of methane-oxidisers and this idea will be developed later in this section.

4. THE RuBP CARBOXYLASE OF METHYLOCOCCUS CAPSULATUS

The demonstration of RuBP carboxylase in a methylotroph has a number of implications both in a consideration of the evolution of autotrophy and in terms of the overall carbon metabolism of M. capsulatus. Further studies on this enzyme from M. capsulatus were then undertaken. Although a purification of the RuBP carboxylase was achieved (this section, 4e), the inability to obtain a significantly and consistently higher specific activity than that quoted in Table 9 prevented a workable quantity of pure enzyme being prepared. As a consequence, many of

the properties of the M. capsulatus RuBP carboxylase were examined using crude extracts or partially purified enzyme.

The following preliminary studies were done using the soluble fraction of cell-free extract as source of RuBP carboxylase. This was prepared from a large scale batch culture of M. capsulatus (Bath).

a) Effect of enzyme concentration

The activity of RuBP carboxylase was found to increase linearly with increasing concentrations of cell-free extract (Fig. 40).

b) Effect of pH

The variation in specific activity with pH was determined using Tris-HCl buffers (Fig. 41). The optimum pH of the M. capsulatus (Bath) RuBP carboxylase under standard assay conditions was 7.6. As with the R. vannielii (RM5) RuBP carboxylase, activity was very much reduced when assayed in phosphate buffers.

c) Effect of temperature

The effect of assay temperature on the activity of RuBP carboxylase in cell-free extracts of both M. capsulatus (Bath) and the mesophile, M. capsulatus (Foster & Davis) was

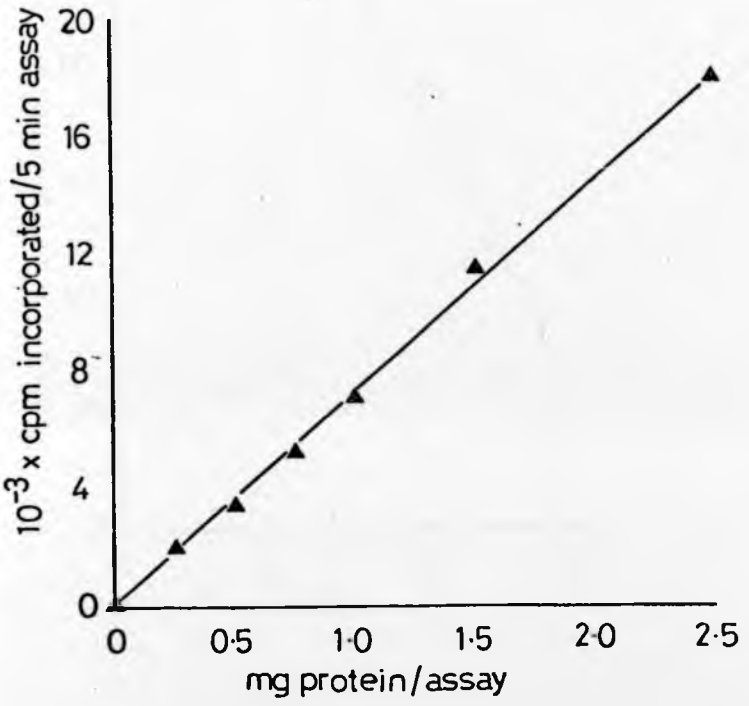

Fig. 40 The effect of increasing concentration of M. capsulatus (Bath) cell-free extract on the activity of RuBP carboxylase

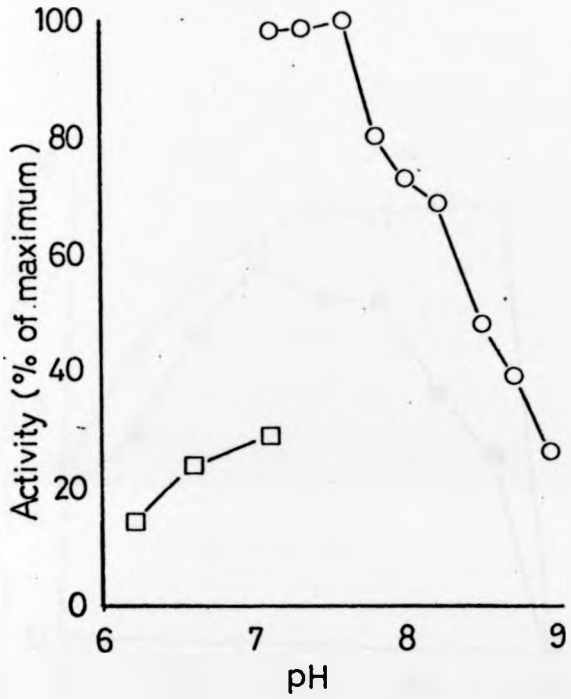

Fig. 41 The effect of pH on the activity of M. capsulatus (Bath) RuBP carboxylase

o - o Tris-HCl buffer

□ - □ Phosphate buffer

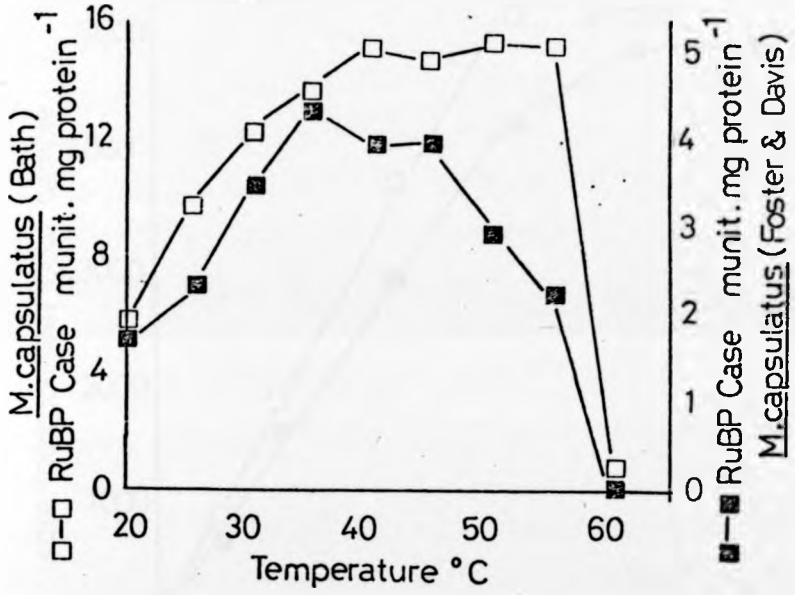

Fig. 42 The effect of temperature on the activity of M. capsulatus (Bath) and M. capsulatus (Foster & Davis) RuBP carboxylase

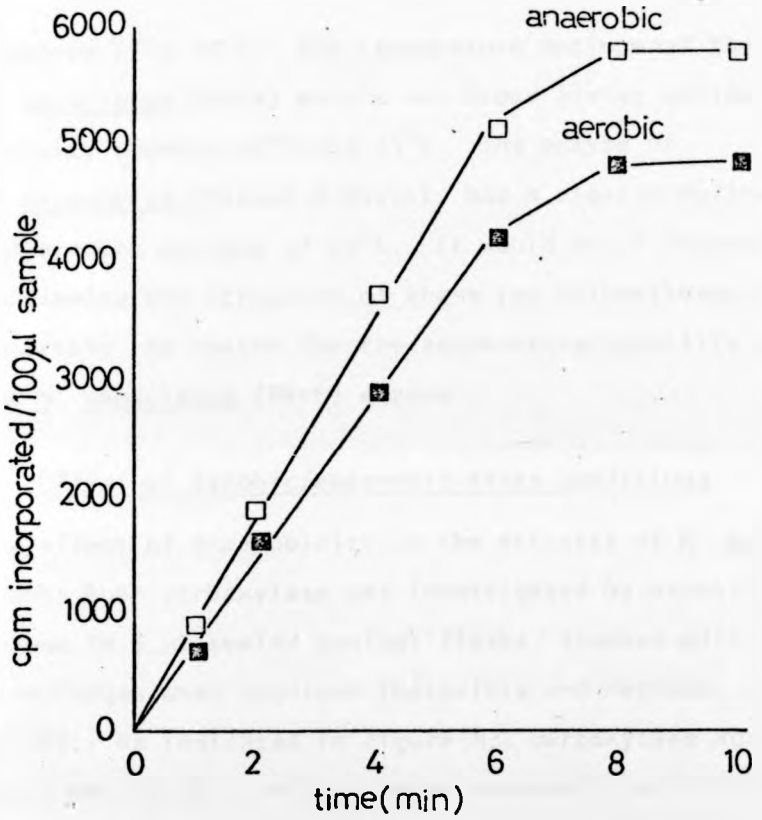

Fig. 43 The activity of M. capsulatus (Bath) RuBP carboxylase when assayed under aerobic and anaerobic conditions

Assay as in Materials and Methods section

2.5 mg protein used per assay of final volume 1 ml.

examined (Fig. 42). The temperature optimum of the M. capsulatus (Bath) enzyme was broad giving maximum activity between 40°C and 55°C. The enzyme of M. capsulatus (Foster & Davis) had a clearly defined temperature optimum of 35°C. It would be of interest to examine the structure of these two carboxylases to ascertain the reason for the temperature stability of the M. capsulatus (Bath) enzyme.

d) Effect of aerobic/anaerobic assay conditions

The effect of anaerobicity on the activity of M. capsulatus (Bath) RuBP carboxylase was investigated by assaying the enzyme in 5 ml sealed conical flasks, flushed with di-nitrogen when required (Materials and Methods, section II.18). As indicated in Figure 43, carboxylase activity was enhanced when assayed under anaerobic conditions, presumably indicative of a competitive oxygenase activity that has been found with all RuBP carboxylases thus far investigated. This effect however, was insufficient to warrant RuBP carboxylase assays being routinely done anaerobically.

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e) The purification of Methylococcus capsulatus (Bath) RuBP carboxylase

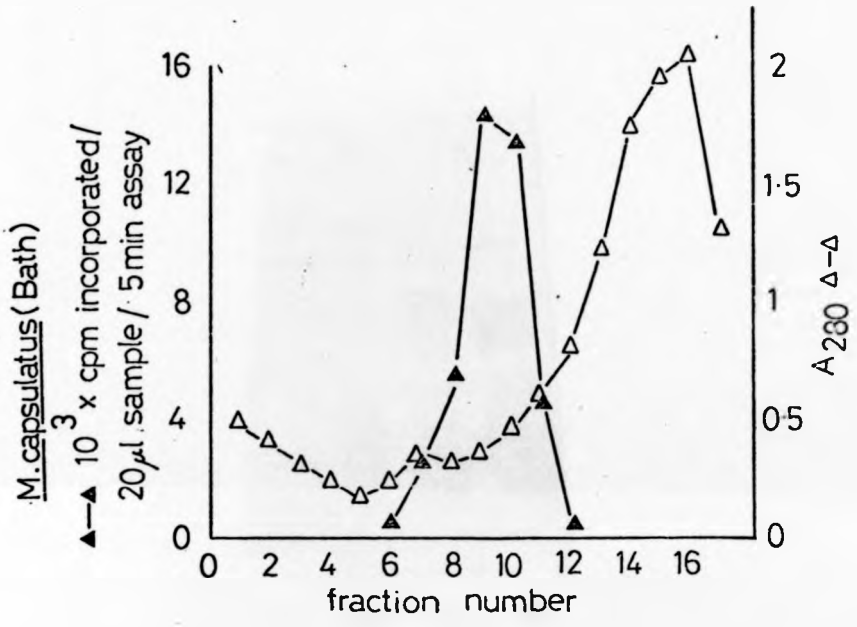
As previously indicated, the major problem involved with the purification of the M. capsulatus RuBP carboxylase was the low specific activity of the enzyme in cell-free extracts. The possible use of sucrose density gradient centrifugation as a one-step purification technique was investigated, but with both M. capsulatus strains, poor separation from bulk proteins resulted (Fig. 44). This did show however, that the enzyme from both strains probably has a similar sedimentation coefficient.

A small quantity of enzyme was purified from M. capsulatus (Bath) using the protocol described in the Materials and Methods section and indicated in Table 13 The purification was followed by SDS-PAGE on exponential (7.5-20% (w/v)) acrylamide gradient gels (Fig. 45). The enzyme gave a single peak of activity on both sucrose gradients (Fig. 46) and after the second, appeared as a single protein band on gels polymerised from 7.5% (w/v) acrylamide (Fig. 47). The procedure gave a 77-fold purification resulting in a final specific activity of 1.26 units.mg.protein^{-1}.

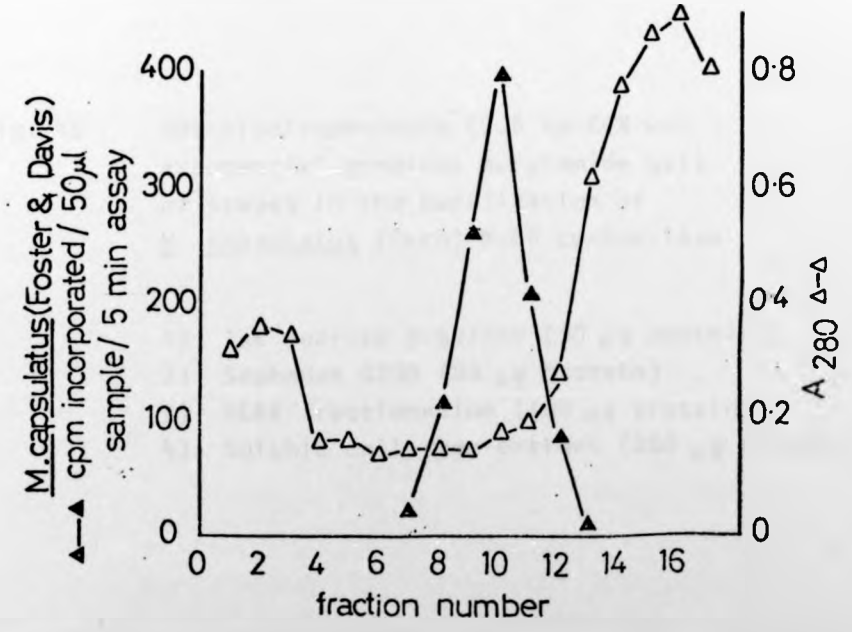
Fig. 44 Sucrose density gradient centrifugation of RuBP carboxylase from (a) M. capsulatus (Bath) and (b) M. capsulatus (Foster & Davis)

3 mg protein as a cell-free extract was applied to each gradient. Centrifugation was for 1½ h in an 8 x 25 fixed angle rotor

a)


M.capsulatus (Bath)
▲—▲ 10³ x cpm incorporated/ 20µl sample/ 5 min assay
A280 △-△
fraction number
0
4
8
12
16
0
0·5
1
1·5
2
0 2 4 6 8 10 12 14 16

b)


M.capsulatus (Foster & Davis)
▲—▲ cpm incorporated/ 50µl sample/ 5 min assay
A280 △-△
fraction number
0
100
200
300
400
0
0·2
0·4
0·6
0·8
0 2 4 6 8 10 12 14 16

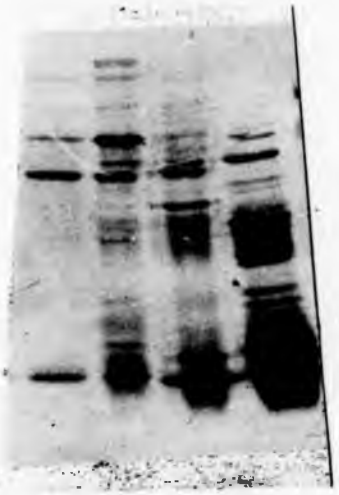


Fig. 45 SDS-electrophoresis (7.5 to 20% w/v exponential gradient acrylamide gel) of stages in the purification of M. capsulatus (Bath) RuBP carboxylase

1) 1st sucrose gradient (50 μg protein)
2) Sephadex G200 (80 μg protein)
3) DEAE fractionation (100 μg protein)
4) Soluble cell-free extract (200 μg protein)

Fig. 46 Purification of M. capsulatus (Bath) RuBP carboxylase. Profile of the second sucrose gradient

Fig. 47 Polyacrylamide gel electrophoretogram of purified M. capsulatus (Bath) RuBP carboxylase (25 μg protein)

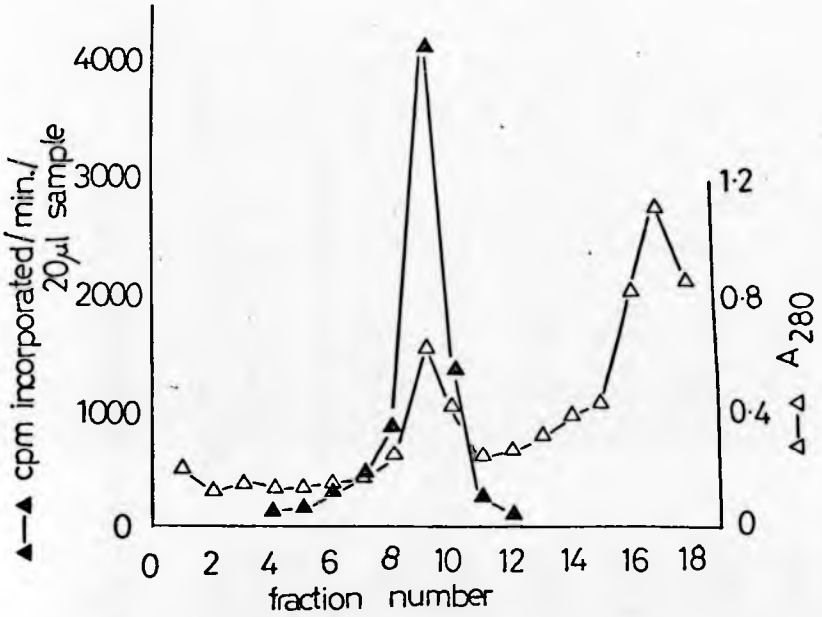

4000
3000
2000
1000
0
1·2
0·8
0·4
0
0 2 4 6 8 10 12 14 16 18
fraction number
▲—▲ cpm incorporated/min./20µl sample
△—△ A280

Table 13 Purification of RuBP carboxylase from M. capsulatus (Bath)

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units.mg protein^{-1})	Yield (%)
Crude cell extract	161	1.77	0.011	100
Soluble (S_{180}) fraction of cell-free extract	104	1.69	0.016	96
Fraction B after DEAE fractionation	22	0.96	0.044	54
Gel filtration on Sephadex G200	5.6	0.77	0.138	44
1st sucrose gradient fractions pooled and dialysed	0.91	0.65	0.595	37
2nd sucrose gradient	0.35	0.44	1.26	25

1 unit of enzyme activity = 1 μmol carbon dioxide fixed.min^{-1}

f) Molecular weight

The molecular weight of the enzyme was determined by gel filtration on a calibrated Sephadex G200 column. Using partially purified enzyme (following first sucrose gradient), a value of 360,000 was obtained (Fig. 48).

g) Quaternary structure

Purified M. capsulatus (Bath) RuBP carboxylase was dissociated by SDS in the presence of 2-mercaptoethanol into two protein subunits as revealed by SDS-PAGE on 10% (w/v) acrylamide slab gels (Fig. 49). These corresponded to the large and small subunits of RuBP carboxylase. The mobility of these proteins in relation to the mobility of dissociated protein standards indicated molecular weights of 48,000 and 14,500 (Fig. 50). No evidence was found suggesting large subunit heterogeneity (see section III.6g; Purohit & McFadden, 1976).

In view of a molecular weight of 360,000, the most likely quaternary structure of the enzyme, assuming equal numbers of both large and small subunits, consists of six large plus six small subunits.

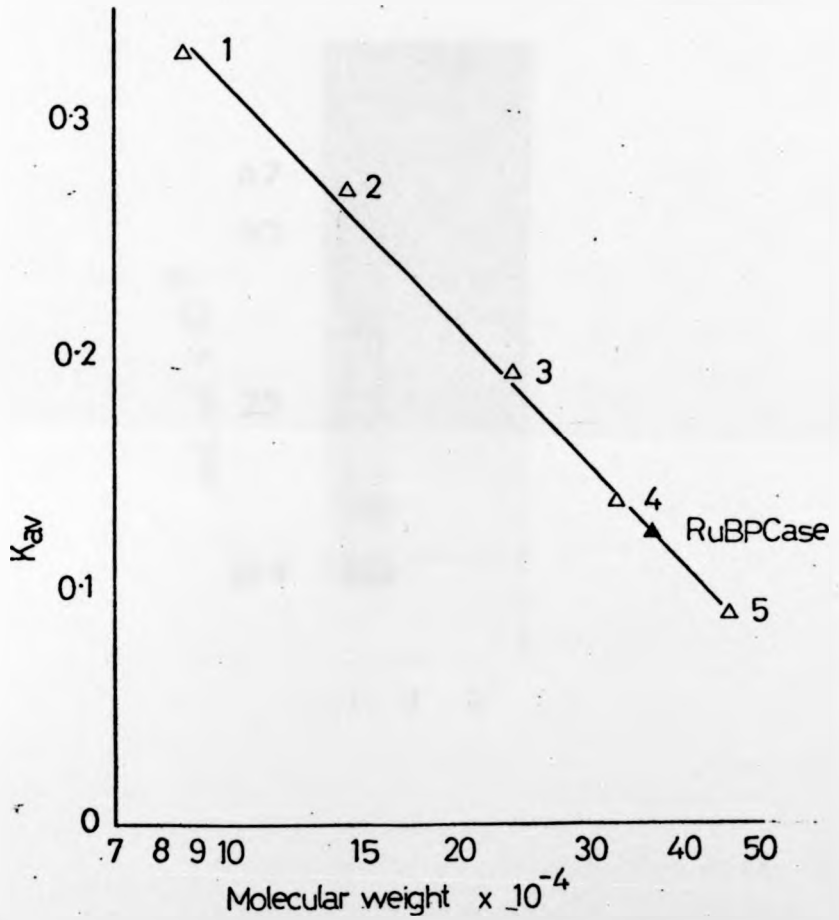


Fig. 48 Molecular weight estimation of M. capsulatus (Bath) RuBP carboxylase on a Sephadex G200 column. Molecular weight standards

(1) alkaline phosphatase; (2) alcohol dehydrogenase; (3) catalase; (4) glutamate dehydrogenase; (5) ferritin

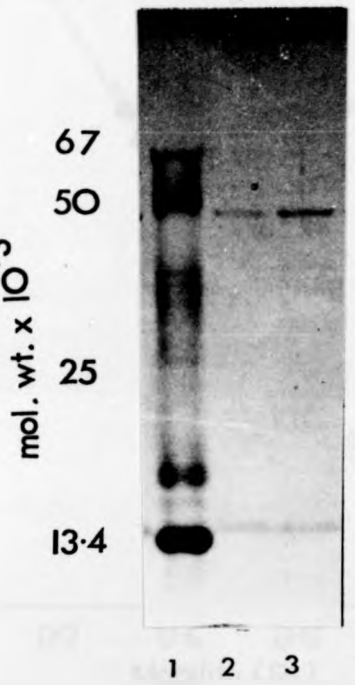


Fig. 49 SDS-electrophoresis (10% w/v acrylamide gel) of M. capsulatus (Bath) RuBP carboxylase

2 : 15μg protein
3 : 25μg protein

1 : Stds

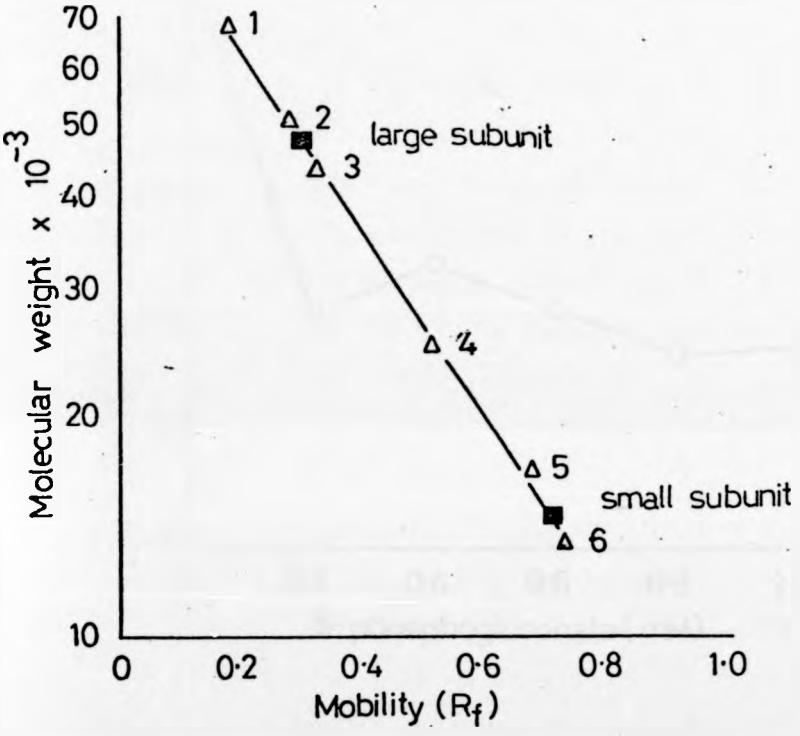


Fig. 50 Determination of the molecular weight of the subunits of M. capsulatus (Bath) RuBP carboxylase by SDS-electrophoresis

1) bovine plasma albumin
2) & 4) γ-globulins
3) ovalbumin
5) myoglobin
6) cytochrome c

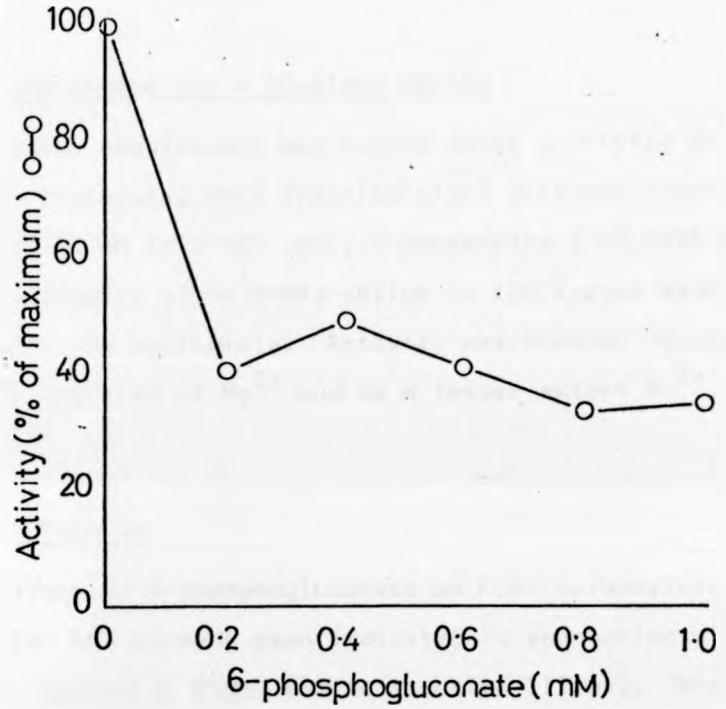


Fig. 51 Effect of 6-phosphogluconate on the activity of M. capsulatus (Bath) RuBP carboxylase

h) Requirement for a divalent cation

The cation requirement was tested using partially purified enzyme (following DEAE fractionation) dialysed overnight against 20 mM Tris-HCl, pH 7.6 containing 5 mM EDTA (Table 14). In the absence of an added cation in the enzyme assay, activity was negligible. Activity was however restored by the addition of Mg^{2+} and to a lesser extent Mn^{2+}, Co^{2+} and Ni^{2+}.

i) Inhibition

The effect of 6-phosphogluconate on RuBP carboxylase activity has already been indicated in connection with the R. vannielii (RM5) enzyme (section III.6f). When included in the assay prior to initiation of the reaction with RuBP, 6-phosphogluconate was an effective inhibitor of purified M. capsulatus (Bath) RuBP carboxylase (Fig. 51). The effect of other inhibitors was not investigated.

j) Effect of substrate concentration

K_m values for RuBP and carbon dioxide were determined using partially purified enzyme (after DEAE fractionation).

Double reciprocal plots of specific activity against increasing RuBP concentration between 10 and 200 μM, gave a K_m value of 71 μM.

Table 14 The effect of various divalent cations on the activity of M. capsulatus (Bath) RuBP carboxylase

Cation in Assay	Specific activity munit.mg protein^{-1}
none	0.07
Mg^{2+}	38.7
Ni^{2+}	7.8
Co^{2+}	10.4
Zn^{2+}	0.1
Mo^{2+}	1.5
Mn^{2+}	6.5
Ca^{2+}	0.5

Assay as in Materials and Methods section using DEAE fractionated cell-free extract as source of enzyme

Double reciprocal plots of specific activity against carbon dioxide concentration when provided as bicarbonate under an air atmosphere, gave a K_m value for total carbon dioxide of 21 mM. This very high value presumably indicates that the enzyme has not been fully activated (Lorimer et al., 1976) and may mean that the specific activities ascribed to the enzyme above, have not been measured under optimum conditions. Further investigation into the activation and substrate kinetics of the M. capsulatus (Bath) RuBP carboxylase is therefore warranted.

k) RuBP oxygenase activity

The ability of all RuBP carboxylases thus far examined to function also as oxygenases, has been indicated in the General Introduction. With both DEAE fractionated cell-free extract and partially purified RuBP carboxylase (after first sucrose gradient) from M. capsulatus (Bath), RuBP dependent oxygen uptake was evident (Table 15).

The specific activity of the oxygenase reaction catalysed by partially purified enzyme was 2.3 nmol oxygen consumed.min^{-1}.mg $protein^{-1}$. This corresponds to 0.057 µmol oxygen consumed.min^{-1}.unit of RuBP carboxylase activity, which is twice the value reported for A. eutropha by Purohit and McFadden (1977). This oxygenase activity is

Table 15 RuBP stimulated oxygen uptake by DEAE fractionated cell-free extract and partially purified RuBP carboxylase of *M. capsulatus* (Bath)

Source of Enzyme	Specific activity nmol O_2 consumed. min^{-1}.mg $protein^{-1}$
DEAE fractionated cell-free extract. Fraction B	0.15
Partially purified RuBP carboxylase (after first sucrose gradient)	2.3
Boiled, partially purified RuBP carboxylase	0

in accord with the higher RuBP carboxylase activity that was measured in the absence of oxygen (this section, 4d).

1) Concluding remarks on the RuBP carboxylase of Methylococcus capsulatus (Bath)

The results described above indicate that the structure and properties of the M. capsulatus (Bath) RuBP carboxylase are not atypical. A molecular weight of 360,000 qualifies this enzyme as an 'Intermediate' class of RuBP carboxylase (Anderson et al., 1968; McFadden, 1973), whilst the presence of small subunits in the enzyme and the inhibition by 6-phosphogluconate have previously only been properties of the 'Large' class of carboxylases. In conjunction with the results for the R. vannielii (RM5) enzyme, this suggests that this crude basis for division into classes may not be broadly applicable.

It is of interest that the M. capsulatus (Bath) enzyme is neither very primitive (i.e. low molecular weight and no small subunits as in the R. rubrum RuBP carboxylase) nor relatively advanced (i.e. molecular weight of around 500,000 with L_8S_8 structure as in Chromatium D, Thiobacillus, etc.). The implications that this has on the possible evolution of autotrophy, will be discussed in the conclusion to this section.

5. C_3-CARBOXYLASES PRESENT IN METHYLOCOCCUS CAPSULATUS (BATH)

Before assessing the importance of the RuBP carboxylase *in vivo*, it was necessary to determine other carboxylase activities that may be present in *M. capsulatus* (Bath). Several types of carboxylase have been found in methylotrophs and one, phosphoenolpyruvate (PEP) carboxylase, is an integral part of the serine pathway for carbon assimilation, found in type II methylotrophs (Large *et al.*, 1962; Anthony, 1975).

The activities of the three C_3-carboxylases that were detectable in extracts of *M. capsulatus* (Bath) are shown in Table 16. For comparative purposes, these enzymes were also assayed in crude cell extracts of *M. trichosporium* (OB3b), a type II methylotroph and *M. methanica* (S1), a type I methylotroph.

The C_3-carboxylases in all three organisms were of similar low activity except for PEP carboxylase in *M. trichosporium* (OB3b). The relatively high activity in OB3b would be expected since this organism assimilates its carbon via the serine pathway. The low activity of these enzymes in *M. capsulatus* (Bath) presumably indicates that a complete serine cycle does not contribute greatly to carbon assimilation during growth on methane. In conclusion,

Table 16 C_3-carboxylase activities present in cell-free extracts of M. capsulatus (Bath), M. trichosporium (OB3b) and M. methanica.

Enzyme	Specific activity.(munit.mg protein^{-1})		
	M. capsulatus (Bath)	M. trichosporium (OB3b)	M. methanica
pyruvate carboxylase	0.15	0.20	1.7
phosphoenol pyruvate (PEP) carboxylase	1.0	14.6	2.9
PEP carboxykinase			
GDP dependent	0.46	0.70	0.80
ADP dependent	0.61	2.7	1.9
IDP dependent	0.34	0.93	0.72

Assays as in Materials and Methods section.

Table 16 C_3-carboxylase activities present in cell-free extracts of M. capsulatus (Bath), M. trichosporium (OB3b) and M. methanica.

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PEP carboxykinase			
GDP dependent	0.46	0.70	0.80
ADP dependent	0.61	2.7	1.9
IDP dependent	0.34	0.93	0.72

Assays as in Materials and Methods section.

although a true appraisal of the individual specific activities of these enzymes requires their purification (Sahl and Truper, 1977), it can be inferred that C_3-carboxylation in M. capsulatus (Bath) is not significant in terms of net carbon fixation.

6. THE ASSIMILATION OF CARBON DIOXIDE BY INTACT CELLS OF METHYLOCOCCUS CAPSULATUS (BATH)

The presence of the key enzymes of the Calvin cycle in cell-free extracts of M. capsulatus leads one to ask two important questions. Do these enzymes operate in vivo and if so, how is their function integrated into the overall metabolism of the cell? To begin to answer these questions, a study of carbon dioxide fixation by whole cells of M. capsulatus (Bath) was undertaken. For consistency, all the following experiments were done using cells taken from an oxygen-limited chemostat at a dilution rate of $0.15h^{-1}$, grown on AMS-medium unless otherwise indicated.

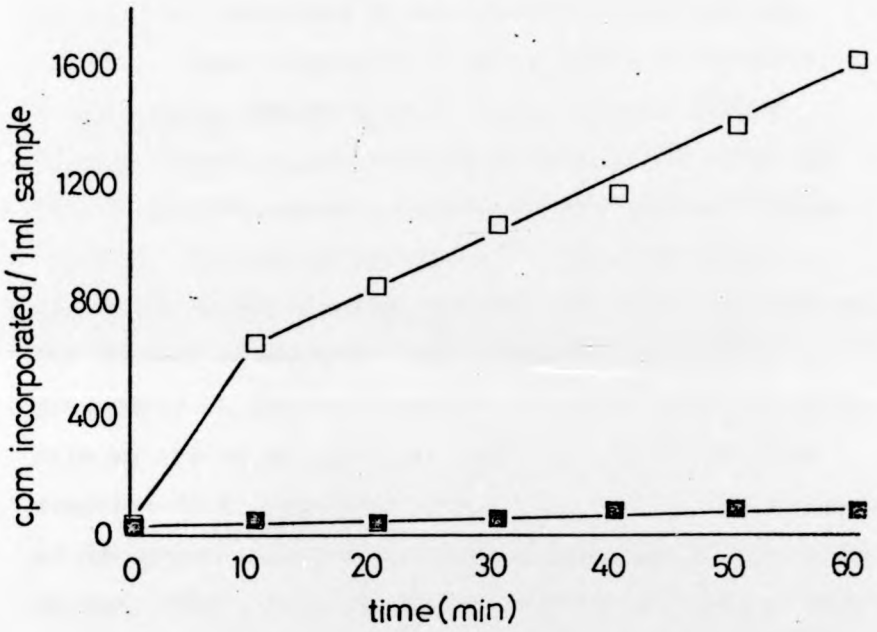


Fig. 52 Incorporation of [^{14}C]-carbon dioxide by intact cells of M. capsulatus (Bath)

Incubation conditions:

■—■ absence of methane

□—□ methane

The uptake of carbon dioxide by whole cells was measured as described in the Materials and Methods section. When incubated in the presence of methane, M. capsulatus (Bath) gave a linear rate of carbon dioxide fixation over a 10 to 60 min period (Fig. 52) with a specific activity of 0.226 μmol carbon dioxide fixed.h^{-1} (mg dry wt of cells)$^{-1}$. No significant uptake of carbon dioxide was observed on incubation in the absence of methane. This methane requirement, presumably as source of energy, would be expected being akin to the requirement of light for carbon dioxide fixation in R. vannielii (RM5). In view of the oxidation of non-growth substrates by M. capsulatus (Stirling and Dalton, 1977), it would be of interest to examine these as potential energy sources to support carbon dioxide fixation.

Assuming a formula for cell material of $C_4H_8O_2N$, which has been demonstrated for a number of methylotrophs (Maclennan et al., 1971; Goldberg et al., 1976), a value may be calculated for the percentage of cell carbon arising from carbon dioxide in the above situation. On the basis of a specific activity of 0.226 μmol.carbon dioxide fixed.h^{-1} (mg dry wt of cells)$^{-1}$ for chemostat grown cells (D = 0.15 h^{-1}), approximately 2.5% (w/w) of the cell carbon originated from carbon dioxide. Because

of uncertainty regarding the carbon dioxide concentration within the chemostat culture, direct measurement of carbon dioxide uptake was not possible, and consequently, resuspension of cells in fresh medium was required. With the physiological effects that this procedure almost certainly has, the value of 2.5% (w/w) may well be very different to the true value. However, it is probable that under the particular growth conditions investigated, carbon dioxide is not a major source of cell carbon. It must also be kept in mind that this calculated value represents all carbon dioxide fixation and not just that mediated by RuBP carboxylase. Even so, the specific activity of RuBP carboxylase in cell-free extracts prepared from the same culture was 6 munit.mg.protein^{-1}, a lower value than that found previously in extracts of batch culture grown cells. This may indicate that under different conditions, where higher RuBP carboxylase activities have been measured, carbon dioxide fixation may assume greater importance.

The high energy requirement in terms of both ATP and NAD(P)H, of carbon dioxide fixation by a Calvin cycle mechanism, has already been indicated. Furthermore, the probable limitation of growth by NAD(P)H in most methylotrophs has been discussed by Anthony (1978). In view of this, it would not be unreasonable to expect a lower rate of carbon dioxide fixation in a culture grown on nitrate or di-nitrogen rather than ammonia as nitrogen source, both of which need to be reduced to the oxidation level of ammonia before assimilation. However, the specific activities of RuBP carboxylase and carbon dioxide fixation measured in chemostat grown M. capsulatus (Bath), with either di-nitrogen or ammonia as nitrogen source are compared in Table 17, and little difference is evident between the two. This may suggest

Table 17 Effect of nitrogen source on the specific activity of RuBP carboxylase and carbon dioxide fixation in M. capsulatus (Bath)

	RuBP carboxylase munit.mg.protein^{-1}	CO_2 fixation rate μmol.h^{-1}.mg dry wt^{-1}
ammonia grown cells	6	0.226
di-nitrogen grown cells	7	0.243

that the availability of reducing power does not significantly influence carbon dioxide fixation during growth of M. capsulatus (Bath) on methane.

It was shown earlier in this section (4j), that the in vitro K_m of the M. capsulatus (Bath) RuBP carboxylase for carbon dioxide (21 mM), was too high to allow for carbon dioxide fixation under physiological conditions. A similar situation has been reported for plant RuBP carboxylases and yet it was found that the K_m for carbon dioxide uptake by intact chloroplasts was some 20-fold smaller than that of the isolated enzyme (Jensen and Bassham, 1966). The K_m for carbon dioxide fixation by intact cells of M. capsulatus (Bath) was determined using increasing concentrations of [^{14}C]-carbon dioxide. Double reciprocal plots of specific activity against carbon dioxide concentration gave a value for 'total carbon dioxide' of 0.86 mM (Fig. 53), considerably less than the in vitro K_m of RuBP carboxylase and closer to the likely physiological carbon dioxide concentration.

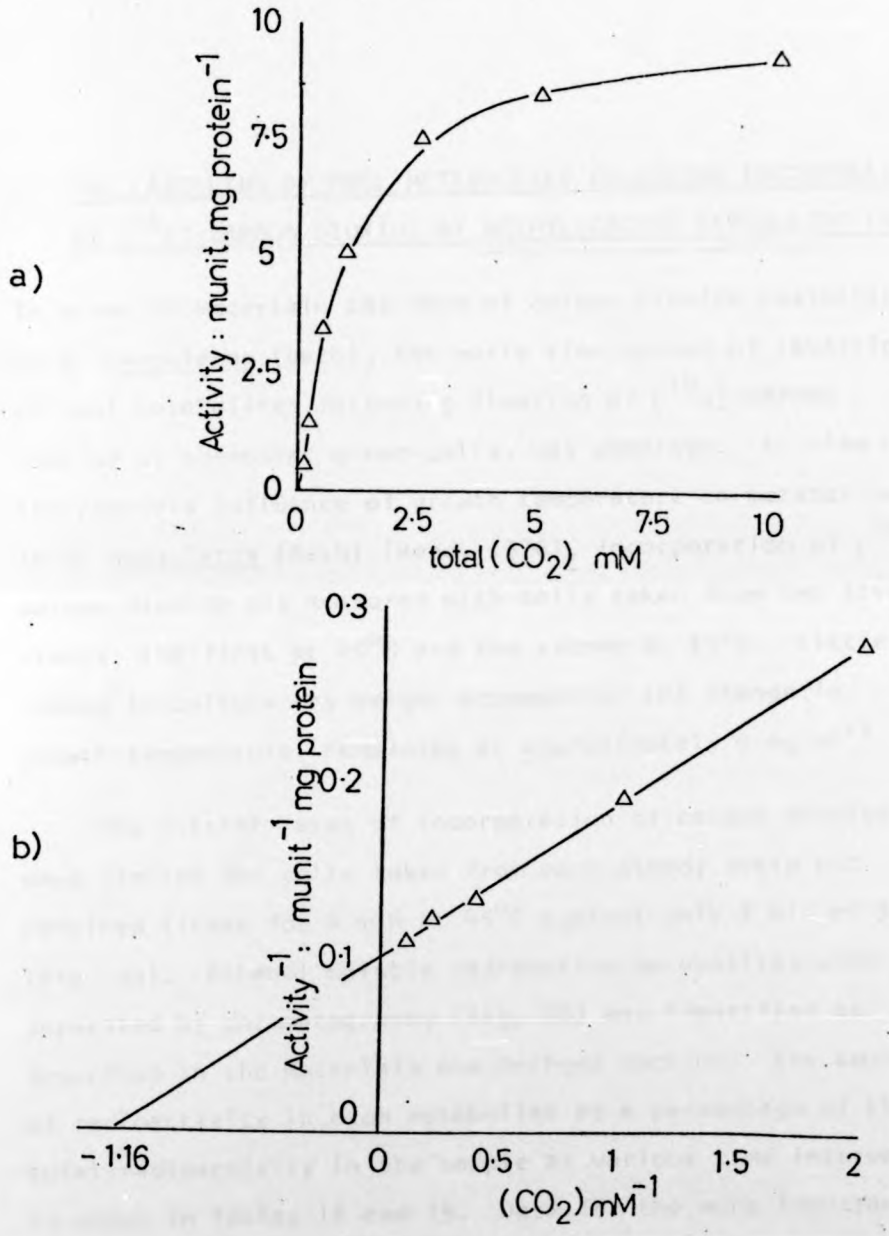


Fig. 53 (a) Effect of carbon dioxide concentration on its incorporation by intact cells of M. capsulatus (Bath) and
(b) Determination of K_m (carbon dioxide)

7. THE LABELLING OF POOL METABOLITES FOLLOWING INCORPORATION OF [^{14}C]-CARBON DIOXIDE BY METHYLOCOCCUS CAPSULATUS (BATH)

In order to ascertain the fate of carbon dioxide assimilated by M. capsulatus (Bath), the early time course of labelling of pool metabolites following fixation of [^{14}C]-carbon dioxide by chemostat grown cells, was examined. In view of the possible influence of growth temperature on metabolism in M. capsulatus (Bath) (Reed, 1976), incorporation of [^{14}C]-carbon dioxide was measured with cells taken from two steady states, the first at 45°C and the second at 35°C. Little change in culture dry weight accompanied the change in growth temperature, remaining at approximately 4 mg.ml^{-1}.

The initial rates of incorporation of carbon dioxide were similar for cells taken from each steady state but remained linear for 4 min at 45°C against only 2 min at 35°C (Fig. 54). Ethanol soluble radioactive metabolites were separated by chromatography (Fig. 55) and identified as described in the Materials and Methods section. The amount of radioactivity in each metabolite as a percentage of the total radioactivity in the sample at various time intervals, is shown in Tables 18 and 19. Data for the more important metabolites is also presented graphically in Figures 56 and 57

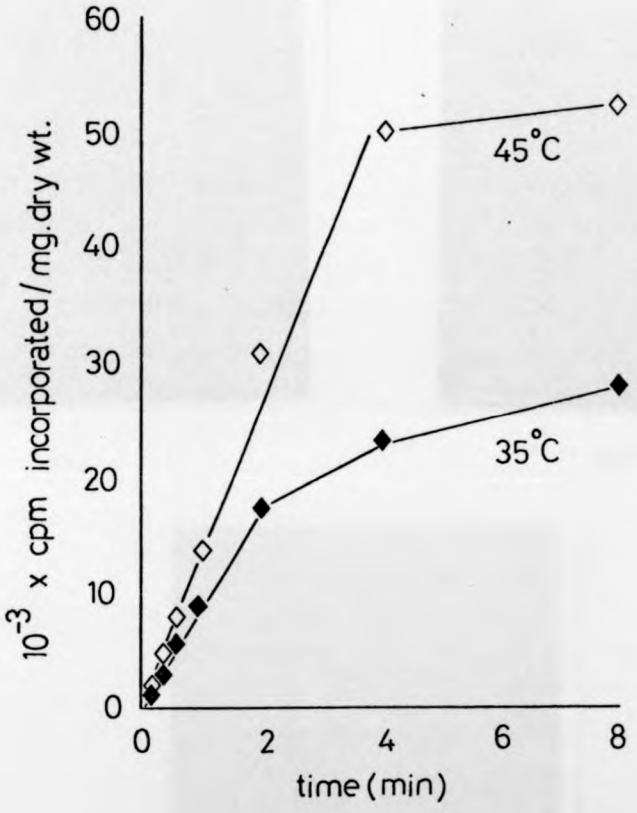


Fig. 54 Effect of temperature on the rate of incorporation of [^{14}C]-carbon dioxide by M. capsulatus (Bath)

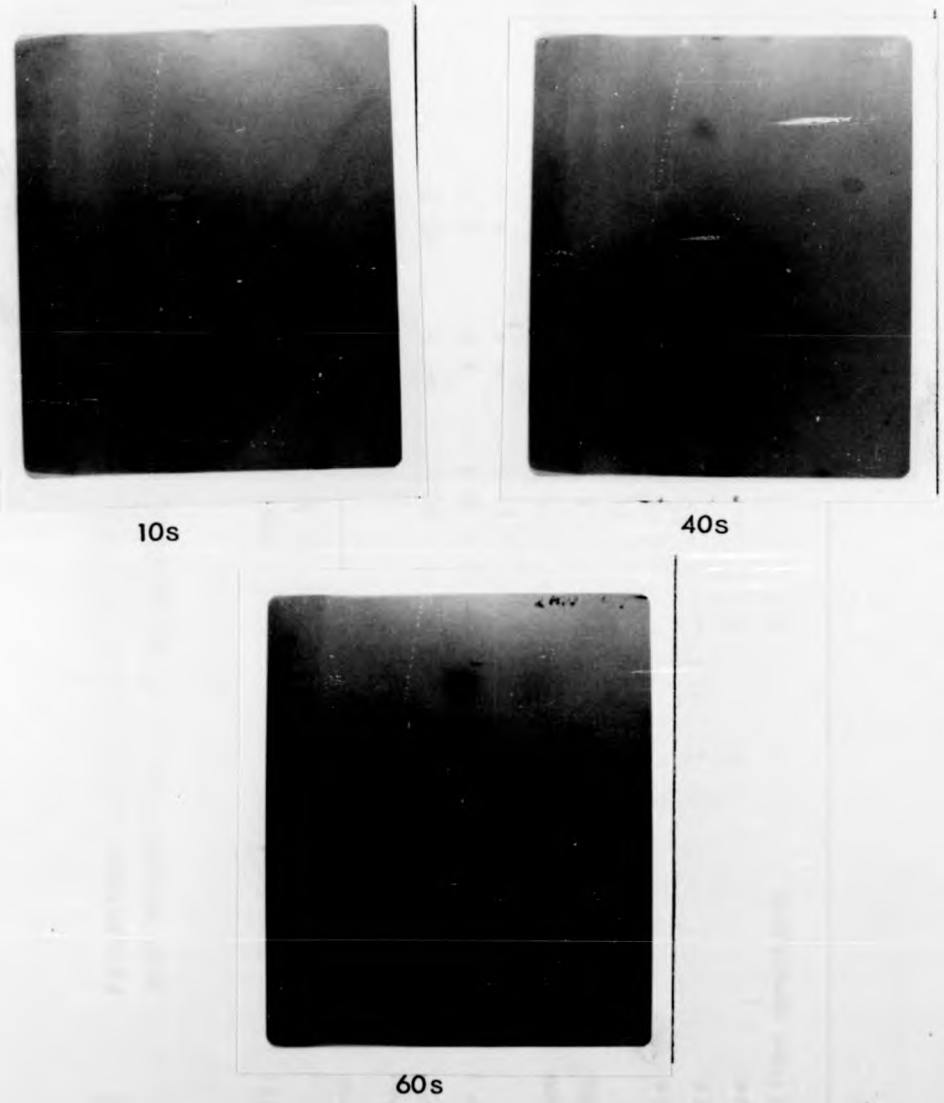


Fig. 55 Two dimensional chromatography of the products of [^{14}C]-carbon dioxide incorporation by M. capsulatus (Bath)

Table 18 Percentage incorporation of [^{14}C]-carbon dioxide into pool metabolites of M. capsulatus (Bath) at 35°C

Metabolite /sample time	Percentage of radioactivity in sample				
	10s	20s	40s	60s	2 min
3-phosphoglycerate	11.4	7.1	7.2	10.4	11.4
aspartate	51.2	40.7	45.3	41.7	42.0
serine + glycine	17.4	12.5	8.1	7.8	5.7
malate	20.0	10.9	11.4	10.3	8.3
phosphoenol pyruvate	-	7.3	4.1	5.5	2.6
other phosphate esters	-	5.6	6.7	6.7	7.2
alanine	-	10.1	10.5	7.8	5.3
succinate	-	5.8	3.1	3.2	2.8
glutamate	-	-	3.6	4.0	7.3
threonine	-	-	-	2.5	1.6
unidentified compounds	-	-	-	0.1	5.8

Table 19 Percentage incorporation of [^{14}C]-carbon dioxide into pool metabolites of M. capsulatus (Bath) at 45°C

Metabolite /sample time	Percentage of radioactivity in sample					
	10s	20s	40s	60s	2 min	4 min
3-phosphoglycerate	27.3	13.0	20.1	13.7	15.6	4.5
aspartate	37.7	26.0	23.5	15.8	9.3	3.5
citrate	14.6	8.5	7.4	7.1	5.3	3.3
malate	20.4	18.2	15.8	20.5	15.6	8.2
phosphoenol pyruvate	-	5.9	4.9	3.8	2.8	1.5
other phosphate esters	-	6.5	4.5	9.7	6.5	6.3
glutamate	-	8.4	11.4	17.4	26.7	27.5
alanine	-	7.6	7.4	6.9	6.9	5.6
succinate	-	5.9	5.0	5.1	5.2	2.5
glutamine	-	-	-	-	2.0	2.7
leu/Ileu/Phe	-	-	-	-	4.3	28.6
threonine	-	-	-	-	-	1.5
serine + glycine	-	-	-	-	-	1.9
unidentified compounds	-	-	-	-	-	2.4

Fig. 56 Distribution of label incorporated from [^{14}C]-carbon dioxide by M. capsulatus (Bath) at 45°C

□ - □ 3-phosphoglycerate

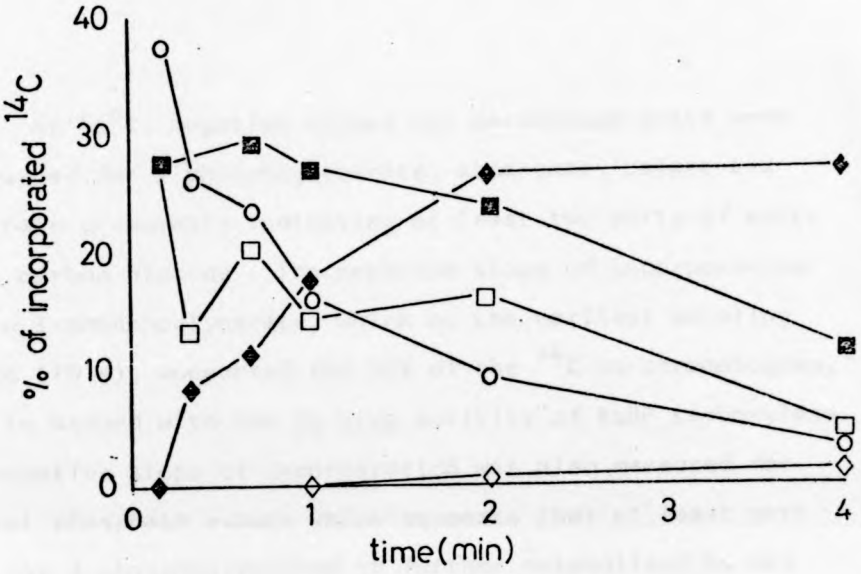
■ - ■ all phosphate esters

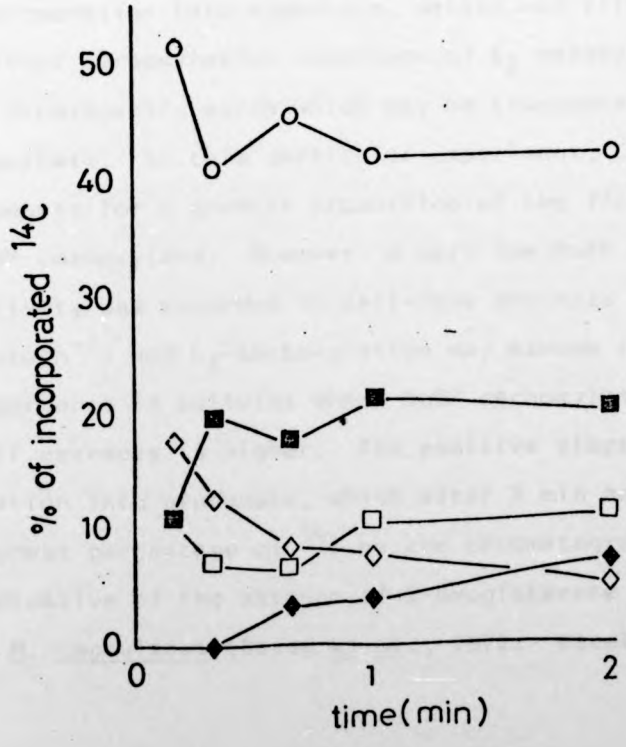
o - o aspartate

◆ - ◆ glutamate

◇ - ◇ serine + glycine

Fig. 57 Distribution of label incorporated from [^{14}C]-carbon dioxide by M. capsulatus (Bath) at 35°C


40
30
20
10
0
% of incorporated ^{14}C
0
1
2
3
4
time(min)


50
40
30
20
10
0
% of incorporated ^{14}C
0
1
2
time(min)

At 45°C, negative slopes for percentage plots were recorded for 3-phosphoglycerate, aspartate, malate and citrate presumably indicating at least two ports of entry for carbon dioxide. The negative slope of incorporation into 3-phosphoglycerate, which at the earliest sampling time (10 s), accounted for 27% of the ^{14}C on chromatogram, is in accord with the *in vivo* activity of RuBP carboxylase. A negative slope of incorporation was also measured for total phosphate esters which suggests that at least part of the 3-phosphoglycerate is further metabolised by way of RMP/Calvin cycle reactions. The negative slopes of incorporation into aspartate, malate and citrate probably reflect carboxylation reactions of C_3 metabolites yielding C_4 dicarboxylic acids which may be transaminated to aspartate. In this particular experiment, C_3-carboxylation accounts for a greater proportion of the fixed ^{14}C than RuBP carboxylase. However, a very low RuBP carboxylase activity was recorded in cell-free extracts (6 munit.mg. protein^{-1}) and C_3-carboxylation may assume relatively less importance in cultures where RuBP carboxylase activity in cell extracts is higher. The positive slope of incorporation into glutamate, which after 2 min accounts for the highest percentage of ^{14}C on the chromatograms, is probably indicative of the absence of 2-oxoglutarate dehydrogenase in *M. capsulatus* (Davey *et al.*, 1972; Patel *et al.*, 1975).

The lack of substantial labelling of tricarboxylic acid cycle intermediates is also suggestive of this enzyme deficiency. Of particular interest is the low percentage of label appearing in serine and glycine, compounds which were heavily labelled in the experiments of Reed (1976), from [^{14}C]-formate. This would seem to argue against a serine pathway operating as a cyclic series of reactions for carbon assimilation in M. capsulatus (Bath) during growth at 45^{o}C, and also indirectly suggests, that formate carbon is not only assimilated as carbon dioxide.

The labelling pattern of the culture grown at 35^{o}C was very different to that at 45^{o}C. At all times, aspartate accounted for the highest percentage of radioactivity on the chromatograms, being over 50% at 10 s and having a negative slope of incorporation. Negative slopes of incorporation were also recorded for serine plus glycine and 3-phosphoglycerate, though these on a percentage basis, were of far less significance. This early labelling of serine and glycine may be due to 3-phosphoglycerate being converted to serine by oxidation and transamination rather than being further metabolised via a series of phosphate esters as part of a RMP/Calvin cycle. However, in view of the heavy labelling of aspartate, the labelling of serine and glycine may also indicate increased importance of C_3-carboxylations as part of a serine cycle of reactions.

The percentage distribution curves of assimilated carbon with time do not indicate the absolute amount of ^{14}C per metabolite and consequently do not allow for a direct comparison of labelling at 45°C and 35°C. Figure 58 a,b,c show the quantitative values of radioactivity per metabolite for aspartate, 3-phosphoglycerate and serine plus glycine, at both 35°C and 45°C. This shows that the actual rate of incorporation into serine plus glycine differs little between the two temperatures which would not be expected if the early labelling of serine plus glycine at 35°C reflected a shift in metabolism from an RMP cycle to a serine cycle. There is clearly however, more ^{14}C assimilated into aspartate at 35°C than at 45°C, and less into 3-phosphoglycerate. This indicates that the shift in growth temperature from 45°C to 35°C has effectively increased the importance of C_3-carboxylation reactions. Whether this represents a major change in the pathways of carbon metabolism or simply indicates different temperature optima for the carboxylase enzymes, awaits further investigation. It is however of interest in view of the altered pattern of metabolite labelling from [^{14}C]-methanol and formate, on change of growth temperature (Whittenbury *et al.*, 1976). This will be further discussed at the end of this section, when considering the integration of RuBP carboxylase and phosphoribulokinase into the carbon metabolism of *M. capsulatus* (Bath).

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Fig. 58a Incorporation of label from [^{14}C]-carbon dioxide into aspartate by M. capsulatus (Bath)

Fig. 58b Incorporation of label from [^{14}C]-carbon dioxide into 3-phosphoglycerate by M. capsulatus (Bath)

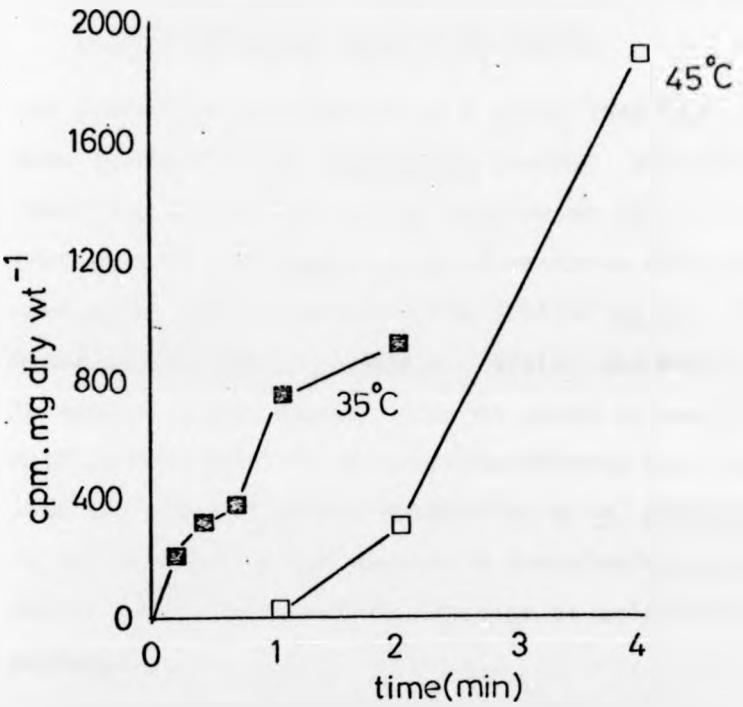


Fig. 58c Incorporation of label from [^{14}C]-carbon dioxide into serine + glycine by M. capsulatus (Bath)

8. THE STATUS OF RuBP CARBOXYLASE AND THE CALVIN CYCLE IN METHYLOCOCCUS CAPSULATUS (BATH)

The preceding experiments have shown that RuBP carboxylase does function in M. capsulatus (Bath). The pattern of labelling from [^{14}C]-carbon dioxide at 45^{o}C is similar to that reported for many types of bacteria with Calvin cycles, when grown non-autotrophically (Fuller et al., 1961; Anderson and Fuller, 1967a,b; Slater and Morris, 1973b). In answering the second question posed on page 218, of how RuBP carboxylase and phosphoribulokinase are integrated into the overall carbon metabolism of M. capsulatus (Bath), it is tempting to ask whether a functioning Calvin cycle can be maintained in this organism as sole carbon assimilation pathway?

RuBP carboxylase and phosphoribulokinase apart, the only other enzyme(s) of the Calvin cycle which are not already present in M. capsulatus (Bath) as part of the RMP pathway, are fructose and sedoheptulose bisphosphatases. However, it is possible to construct a hypothetical variant of the Calvin cycle not involving sedoheptulose bisphosphatase (Fig. 1 b) and in R. palustris, both of these activities reside with a single enzyme (Springgate and Stachow, 1972). A low level of fructose bisphosphatase was detected in cell-free extracts of M. capsulatus (Bath) with

a specific activity of 4.8 munit.mg.protein^{-1}. It is evident therefore, that although some enzymes are present apparently at low specific activity, the basic machinery of the Calvin cycle is present in this organism.

Formate, ethanol, hydrogen and ammonia are all oxidised by M. capsulatus (Bath) (R. Whittenbury, personal communication) and may be considered as potential electron donors for autotrophic growth on carbon dioxide. However, none of these compounds in combination with carbon dioxide, supported detectable growth. Although it remains possible that growth requirements are very specific and may not have been satisfied experimentally, this does suggest that carbon dioxide cannot be utilised as the sole source of carbon. This is also inferred indirectly by there being no report of Methylococcus having been isolated as a carbon dioxide utilizing autotroph.

There are essentially two explanations for this situation. Firstly, there may be no suitable electron donor which can provide sufficient energy and reducing power to support a Calvin cycle. M. capsulatus (Bath) is very restricted in terms of suitable carbon sources for growth, with methane its prime substrate. It would not therefore be unreasonable to suggest that possible reductants such as formate and hydrogen, cannot be utilized

efficiently enough to generate the requisite amount of energy to support all cellular functions whilst maintaining an energy consuming Calvin cycle.

An alternative to this hypothesis is based on the finding that the specific enzymes of the Calvin cycle in M. capsulatus (Bath), are all present at very low activity in extracts of the methane grown organism, and as such, would not be expected to support a Calvin cycle as sole carbon assimilation pathway. Most organisms have higher levels of these enzymes when grown autotrophically than when grown non-autotrophically (Lascelles, 1960; McFadden and Tu, 1967; Kuehn and McFadden, 1968; Tabita and McFadden, 1974a). It may therefore be that these enzymes in M. capsulatus (Bath) are present at a constitutively low level and their specific activities cannot be raised to allow for the utilisation of carbon dioxide. The constitutive nature of RuBP carboxylase has already been implied by its remaining at a constant specific activity when M. capsulatus (Bath) is switched from growth on AMS-medium to nitrogen-free medium (this section, subsection 6). This idea is particularly attractive in view of the mode of regulation of RuBP carboxylase in other organisms.

The high activity of this enzyme that accompanies the autotrophic growth of Ps. oxalaticus on formate, is believed to arise either as a result of induction of enzyme synthesis by formate or a derivative (Blackmore and Quayle, 1968), from de-repression of enzyme synthesis where the heterotrophic substrate or derivate is the repressor (Dijkhuizen et al., 1978) or from the operation of both effects. A derepression/repression mechanism has also been proposed for RuBP carboxylase control in R. rubrum (Slater and Morris, 1973a). With the similar nature of methane and carbon dioxide metabolism, a derepression/repression or induction control or RuBP carboxylase in M. capsulatus (Bath) based upon methane, carbon dioxide or a derivative may be considered unlikely. Therefore, it would be difficult to propose a regulatory mechanism which could allow distinction between growth on methane by the RMP pathway and growth on carbon dioxide by a Calvin cycle.

It would be interesting to examine the possibility that methane, or metabolite produced from methane, represses RuBP carboxylase synthesis by depriving a culture of M. capsulatus (Bath) of this substrate and following any change in the specific activity of RuBP carboxylase. If repression is occurring, then an increase in activity may follow in a similar way to the reported increase in carbon

dioxide fixation rate in R. rubrum on malate starvation (Slater and Morris, 1973a).

The evidence presented above therefore suggests that RuBP carboxylase and phosphoribulokinase are either, part of a novel carbon assimilation pathway for C_1 compounds in M. capsulatus (Bath) or else represent an evolutionary transition from Calvin cycle to RMP pathway, or vice versa, and have little importance with respect to the metabolism of this organism.

9. THE METABOLISM OF 2-PHOSPHOGLYCOLLATE IN METHYLOCOCCUS CAPSULATUS (BATH)

The ability of the M. capsulatus (Bath) RuBP carboxylase to function also as an oxygenase forming 3-phosphoglycerate and 2-phosphoglycollate, was indicated earlier in this section (subsection 4k). Enzymes of 2-phosphoglycollate metabolism are generally characteristic of carbon dioxide assimilating autotrophs although several have also been implicated in the serine pathway of carbon assimilation found in methylotrophs, e.g. glycollate oxidase, hydroxypyruvate reductase, glycerate kinase (Anthony, 1975). In view of the presence of hydroxypyruvate reductase in

M. capsulatus (Bath), an investigation into the metabolism of 2-phosphoglycollate by this organism was done.

a) The presence of phosphoglycollate phosphatase

The soluble fraction of cell-free extracts of M. capsulatus (Bath) contained phosphoglycollate phosphatase activity, dependent upon the presence of Mg^{2+}. A specific activity of 77 munit.mg protein^{-1} was measured at the pH optimum of 6.3 (Fig. 59) and double reciprocal plots of specific activity against 2-phosphoglycollate concentration indicated a K_m of 3.4 mM. Although a high value, the K_m for 2-phosphoglycollate with the pea enzyme has been shown to be dependent upon pH and Mg^{2+} concentration (Halliwell, 1976) and the M. capsulatus phosphatase may be similarly affected. To determine the substrate specificity of the M. capsulatus (Bath) enzyme, a partial purification was effected as shown in Table 20, and the active phosphoglycollate phosphatase fractions from the sucrose gradient tested for other phosphatase activities. Appreciable activity was found only with 2-phosphoglycollate (Table 21).

No phosphoglycollate phosphatase activity was found in cell-free extracts of M. methanica (S1), an organism which also possesses the RMP pathway for carbon assimilation but which has no significant RuBP carboxylase activity. The presence of this enzyme in M. capsulatus (Bath) therefore correlates with the presence of RuBP carboxylase/oxygenase.

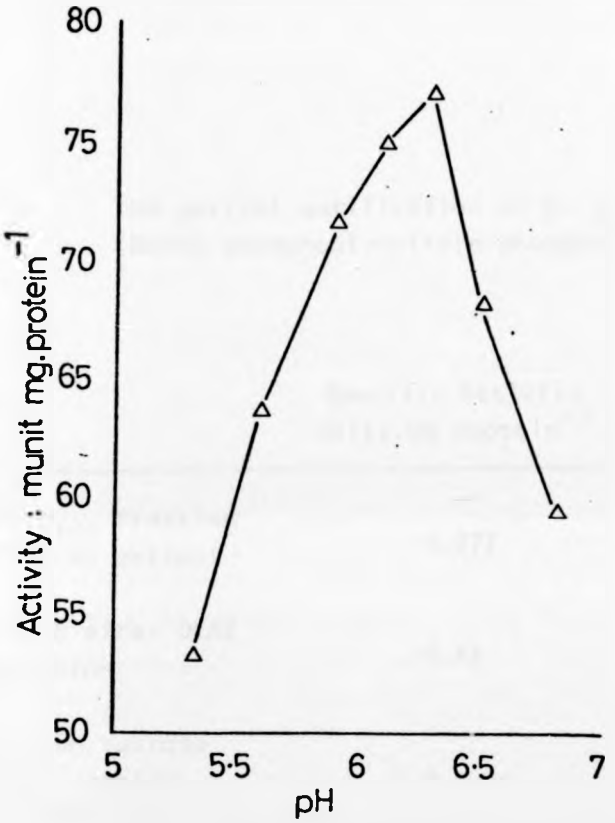


Fig. 59 Effect of pH on the activity of M. capsulatus (Bath) phosphoglycollate phosphatase

Table 20 The partial purification of M. capsulatus (Bath) phosphoglycollate phosphatase

Step	Specific Activity units.mg protein^{-1}	Purifaction factor
soluble S_{180} fraction of cell-free extract	0.077	-
fraction B after DEAE fractionation	0.23	3.0
0.2 to 0.8M sucrose gradient - pooled active fractions	0.48	6.2

Table 21 Substrate specificity of the phosphoglycollate phosphatase from M. capsulatus (Bath)

Substrate	Specific activity units.mg protein^{-1}
2-phosphoglycollate	0.480
3-phosphoglycerate	0.010
fructose 1,6-bisphosphate	-
fructose 6-phosphate	-
glucose 6-phosphate	-
6-phosphogluconate	0.015
ribose 5-phosphate	-
sedoheptulose 7-phosphate	-
sedoheptulose 1,7-bisphosphate	-
phosphoenol pyruvate	0.012

b) The incorporation of [^{14}C]-1-glycollate

The further metabolism of glycollate in M. capsulatus (Bath) was investigated by following the uptake of [^{14}C]-1-glycollate by intact cells as described in the Materials and Methods section.

The incorporation of exogeneous glycollate by methane grown M. capsulatus (Bath) although poor, remained linear for at least 25 min (Fig. 60). This suggests that glycollate is metabolised by this organism and not simply excreted into the growth medium as reported for R. rubrum (Codd and Smith, 1974). The time course of labelling of pool metabolites from [^{14}C]-1-glycollate is indicated in Table 22. Results for the more important metabolites are also presented graphically in Figure 61.

At the earliest sampling time (1 min), the highest percentage (27%) of ^{14}C on the chromatogram was present in serine plus glycine and a negative slope of incorporation with time into these compounds, was evident. After 1 min, phosphate esters only accounted for 6.5% of the fixed ^{14}C and moreover exhibited a positive slope of incorporation of label. A similar positive slope of incorporation was recorded for glutamate. Although not present on chromatograms after 4 min incubation, glycerate accounted for 5.3% of the fixed ^{14}C at 1 min, indicating

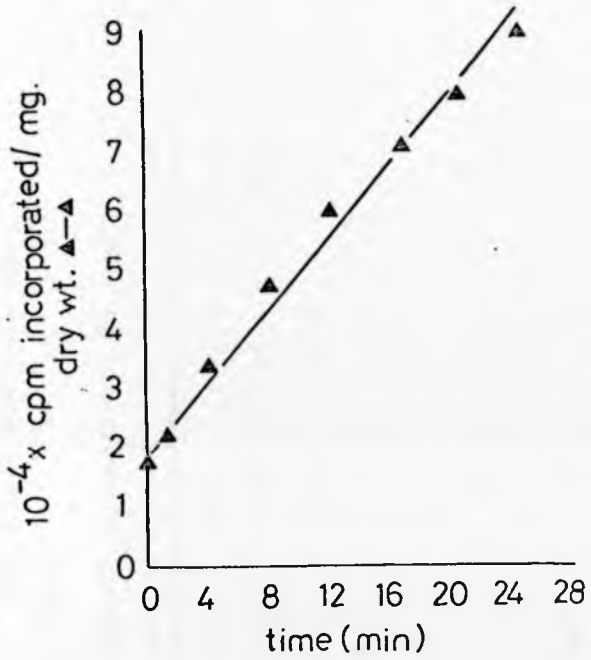


Fig. 60 Incorporation of [^{14}C]-1-glycollate by intact cells of M. capsulatus (Bath)

Table 22 Percentage incorporation of [^{14}C]-I-glycollate into pool metabolites of M. capsulatus (Bath)

Metabolite / sample time	Percentage of radioactivity in sample				
	1 min	4 min	8 min	12 min	16 min
aspartate	6.0	10.3	10.1	12.5	15.3
serine + glycine	27.4	23.9	17.0	15.1	18.5
glutamate	15.3	27.5	22.0	21.0	17.7
glutamine	13.5	3.6	-	-	-
proline	8.9	-	-	-	-
valine	6.8	-	-	-	-
leucine	5.9	-	-	-	-
3-phosphoglycerate	-	4.8	2.9	1.4	-
phosphoenolpyruvate	-	4.0	2.3	1.9	1.7
other phosphate esters	6.5	20.9	28.0	30.6	32.3
unidentified compounds					

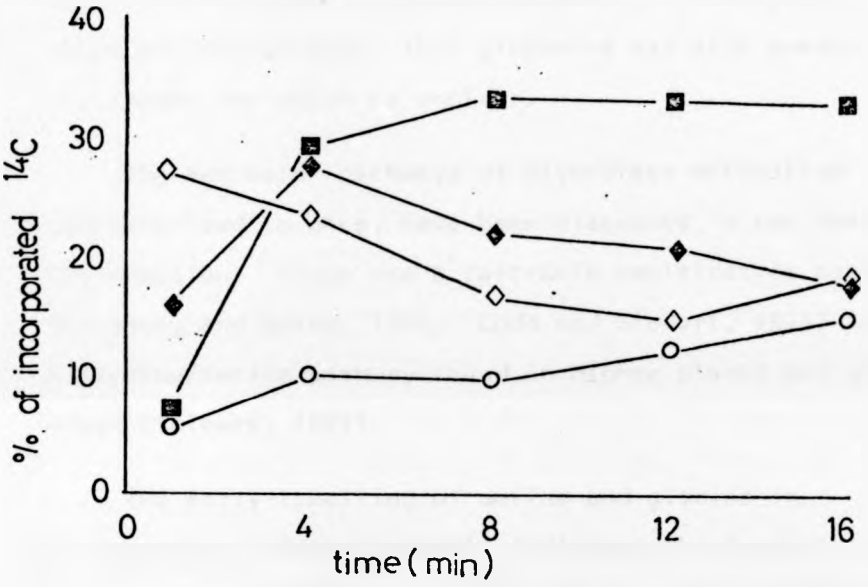


Fig. 61 Distribution label incorporated from [^{14}C]-I-glycollate by M. capsulatus (Bath) at 45°C

O—O aspartate

◇—◇ serine + glycine

◆—◆ glutamate

■—■ phosphate esters

that it was an early labelled metabolite. A negative slope of incorporation into glutamine was also measured, the reason for which is unclear.

The two major pathways of glycollate metabolism characterised to date, have been discussed in the General Introduction. These are a tartronic semialdehyde pathway (Kornberg and Gotto, 1961; Codd and Stewart, 1973) and a glycine/serine pathway found in higher plants and green algae (Tolbert, 1971).

The early labelling of serine and glycine in M. capsulatus (Bath) probably indicates the formation of glycine from glycollate via glyoxylate, the presence of which would not be demonstrated by the chromatography system used. The non-occurence of large amounts of label initially in phosphate esters excludes the tartronic semialdehyde pathway as an important route of glycollate metabolism in this organism as these are the major intermediates of that pathway. The positive slope of incorporation into phosphate esters presumably indicates that label is rapidly transferred from serine and glycine to these compounds, probably via 3-phosphoglycerate.

The absence of label in tricarboxylic acid cycle intermediates, notably malate, suggests that glycollate is not significantly metabolised via a glyoxylate cycle.

Furthermore, this also suggests that the β-hydroxyaspartate pathway (Kornberg and Morris, 1965) is not functional in this organism.

There is no evidence to suggest how a glycine to serine conversion would operate in M. capsulatus (Bath). In higher plants, this reaction involves loss of carbon dioxide and although this mechanism may well occur in M. capsulatus (Bath, it remains possible that a serine transhydroxymethylase (12) type reaction is responsible. This is particularly attractive in view of the early

(12) glycine + $N^{5,10}$ - methylene tetrahydrofolate ⟶ serine + tetrahydrofolate

labelling of serine from [^{14}C]-formate, reported by Whittenbury et al. (1976). Whether these reactions would constitute a serine pathway is at present unclear and will be discussed later in this section.

The probable conversion of serine to 3-phosphoglycerate indicated by the labelling pattern above, provides an explanation for the presence of hydroxypyruvate reductase in M. capsulatus (Bath) (Reed, 1976) in that in higher plants, serine is metabolised to 3-phosphoglycerate via hydroxypyruvate and glycerate in turn.

c) Hydroxypyruvate reductase in M. capsulatus (Bath)

Although the main activity of this enzyme is the reduction of hydroxypyruvate to glycerate, it has also been implicated in the interconversion of glyoxylate and glycollate (Anthony, 1975) as it is able to catalyse the reduction of glyoxylate at about one-tenth of the rate of hydroxypyruvate. The involvement of this enzyme in glycollate metabolism by M. capsulatus (Bath) was therefore investigated.

The soluble fraction of cell-free extracts of M. capsulatus (Bath) was assayed for hydroxypyruvate reductase activity with either hydroxypyruvate or glyoxylate as substrate (Table 23). The specific activity of glyoxylate reduction was approximately 80% of the activity measured with hydroxypyruvate. The relative specific activities with both substrates remained constant over a partial enzyme purification indicating that both activities may reside on the one enzyme.

Double reciprocal plots of specific activity against substrate concentration with the partially pure enzyme gave K_m values of 3.3 mM for glyoxylate and 50 μM for hydroxypyruvate indicating a much higher affinity of the enzyme for hydroxypyruvate. Consequently, although the M. capsulatus (Bath) enzyme is far more responsive to

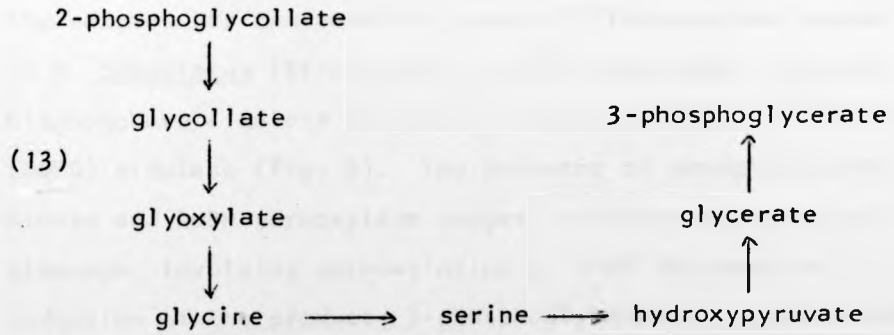
Table 23 Partial purification of Hydroxypyruvate reductase from M. capsulatus (Bath) assayed with either hydroxypyruvate or glyoxylate as substrate

Step	Specific activity units.(mg protein^{-1})	
	Hydroxypyruvate	Glyoxylate
soluble S_{180} fraction of cell free extract	0.041	0.033
fraction A after DEAE fractionation	0.128	0.107
gel filtration on Ultrogel AcA 34	0.255	0.193

glyoxylate than other hydroxypyruvate reductases, the high K_m value in relation to that for hydroxypyruvate argues against a physiological role in glycollate oxidation.

Further study of the enzymes of glycollate metabolism in M. capsulatus (Bath) is required before the precise nature of the pathway may be elucidated. However, the data presented above suggests a pathway of the type shown below (13).

(13)



Pa. denitrificans assimilates C_1 compounds by a Calvin cycle and this organism also has a hydroxypyruvate reductase, the reason for the presence of which is unclear (Bamforth and Quayle, 1977). In view of the results with M. capsulatus (Bath), this enzyme may be involved in the metabolism of phosphoglycollate resulting from RuBP oxygenase activity.

10. OVERALL CARBON METABOLISM OF METHYLOCOCCUS CAPSULATUS (BATH)

The results described above have indicated that RuBP carboxylase does function in the cellular metabolism of M. capsulatus (Bath) although a complete Calvin cycle may not be present. How therefore are the activities of this enzyme and phosphoribulokinase integrated into the major route of carbon assimilation, viz the RMP cycle (Strom et al., 1974), in this organism?

As indicated in the General Introduction, the RMP cycle may be divided into three phases; carbon fixation, cleavage and rearrangement (Quayle and Ferenci, 1978). The enzymes for two possible types of cleavage are present in M. capsulatus (Strom et al., 1974) based upon fructose bisphosphate aldolase or 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (Fig. 5). The presence of phosphoribulokinase and RuBP carboxylase suggest an alternative to this cleavage, involving carboxylation of RuBP followed by reduction of the product, 3-phosphoglycerate to glyceraldehyde 3-phosphate (GAP). These three schemes are indicated in Figure 62. The requirement for reduction of 3-phosphoglycerate entails greater expenditure of energy than is required for fixation of formaldehyde and subsequent cleavage in the 'normal' RMP cycle. This is clearly shown in Table 24

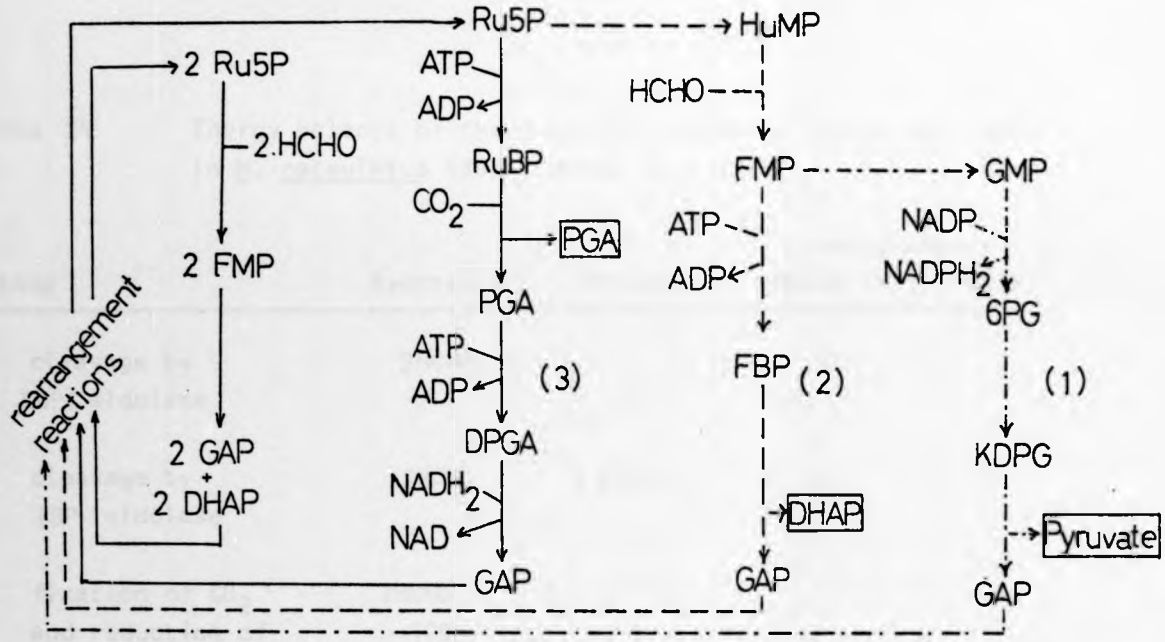


Fig. 62 Possible schemes for carbon fixation and C_6 cleavage in M. capsulatus (Bath)

1) ·-·-·-·→ Cleavage by KDPG aldolase

2) -------→ Cleavage by FBP aldolase

3) ——————→ CO_2 fixation and reduction of PGA

Table 24 Energy balance of three possible schemes of the RMP cycle in M. capsulatus (Bath) shown in Fig. 62

Scheme	Reactants	Product	Energy Change DNAD(P)H	Energy Change DATP
1. cleavage by KDPG aldolase	3HCHO	↑	+1	0
2. cleavage by FBP aldolase	3HCHO	Pyruvate	+1	+1
3. fixation of CO_2 and reduction of 3-phosphoglycerate	2HCHO + $1CO_2$	↓	-1	-1

where the product of each scheme is converted to pyruvate by established glycolytic steps. Obviously, in scheme 3 involving RuBP carboxylase, the ratio of reactants need not be 2 formaldehyde : 1 carbon dioxide, and both the *in vitro* and *in vivo* studies described in this thesis suggest far more cell carbon arises from formaldehyde than from carbon dioxide.

Although the fixation of carbon dioxide rather than formaldehyde is energetically unfavourable, this must be considered with reference to the natural environment of *M. capsulatus*. In this context, the ability to scavenge for a carbon source in addition to methane which has low solubility, may be advantageous and carbon dioxide would be readily available. As discussed in the General Introduction, growth of *M. capsulatus* is probably limited by the availability of NAD(P)H (Anthony, 1978). However, the NAD(P)H required for carbon dioxide fixation by *M. capsulatus* (Bath) could be provided either by the dissimilatory cycle of formaldehyde oxidation (Strom *et al.*, 1974; Fig. 8) or by an ATP dependent reversal of electron flow following hydroxylamine oxidation (Dalton, 1977).

Consideration must also be given to the effect of RuBP oxygenase activity leading to phosphoglycollate and subsequently, glycollate formation. The labelling of

glycollate in M. capsulatus (Bath), after fixation of [^{14}C]-methanol (Reed, 1976), is suggestive of this oxygenase activity occurring in vivo. Studies described earlier in this section have indicated that glycollate is metabolised by M. capsulatus (Bath) although the precise nature of the pathway and particularly the manner of conversion of glycine to serine, is unknown.

Whittenbury et al. (1976) have indicated that formate is not metabolised by the same pathway as methanol in M. capsulatus (Bath). Furthermore, although growth and experimental conditions were different, the pattern of labelling of pool metabolites from [^{14}C]-formate was very different to the labelling pattern from [^{14}C]-bicarbonate reported here. Together, these results suggest that formate is not assimilated in M. capsulatus (Bath) either by a RMP cycle or as carbon dioxide by RuBP carboxylase. The highest percentage of label from [^{14}C]-formate initially appeared in serine and glycine (Whittenbury et al., 1976) and it is therefore not unreasonable to suggest that formate, probably as a tetrahydrofolate derivative, condenses with glycine to give serine as part of the sequence of glycollate metabolising reactions.

The high percentage of label that initially appears in aspartate following uptake of [^{14}C]-bicarbonate by M. capsulatus (Bath), was demonstrated earlier in this section, this probably indicating PEP carboxylase activity. The enzymic machinery may therefore be present in this organism to synthesise a C_4 skeleton from 2-phosphoglycollate (Fig. 63). This would effectively constitute a serine pathway although the question of whether this operates as part of a cyclic series of reactions remains unanswered.

Further elucidation of the pathways of carbon metabolism in M. capsulatus (Bath) must await examination of cell-free extracts for the enzyme activities concerned, in particular tetrahydrofolate formylase, serine transhydroxymethylase and the enzymes necessary to complete a serine cycle.

It has been suggested (Whittenbury et al., 1976) that growth temperature affects the pathway of carbon assimilation in M. capsulatus (Bath). At 45°C, both RMP and serine cycles are considered to operate whilst at 30°C, only the RMP cycle functions. The higher in vivo activity of C_3-carboxylations at 35°C than at 45°C, shown earlier in this section from [^{14}C]-bicarbonate uptake experiments, is not consistent with there being less serine cycle activity at 35°C than at 45°C. However, the higher RuBP carboxylase-oxygenase activity at

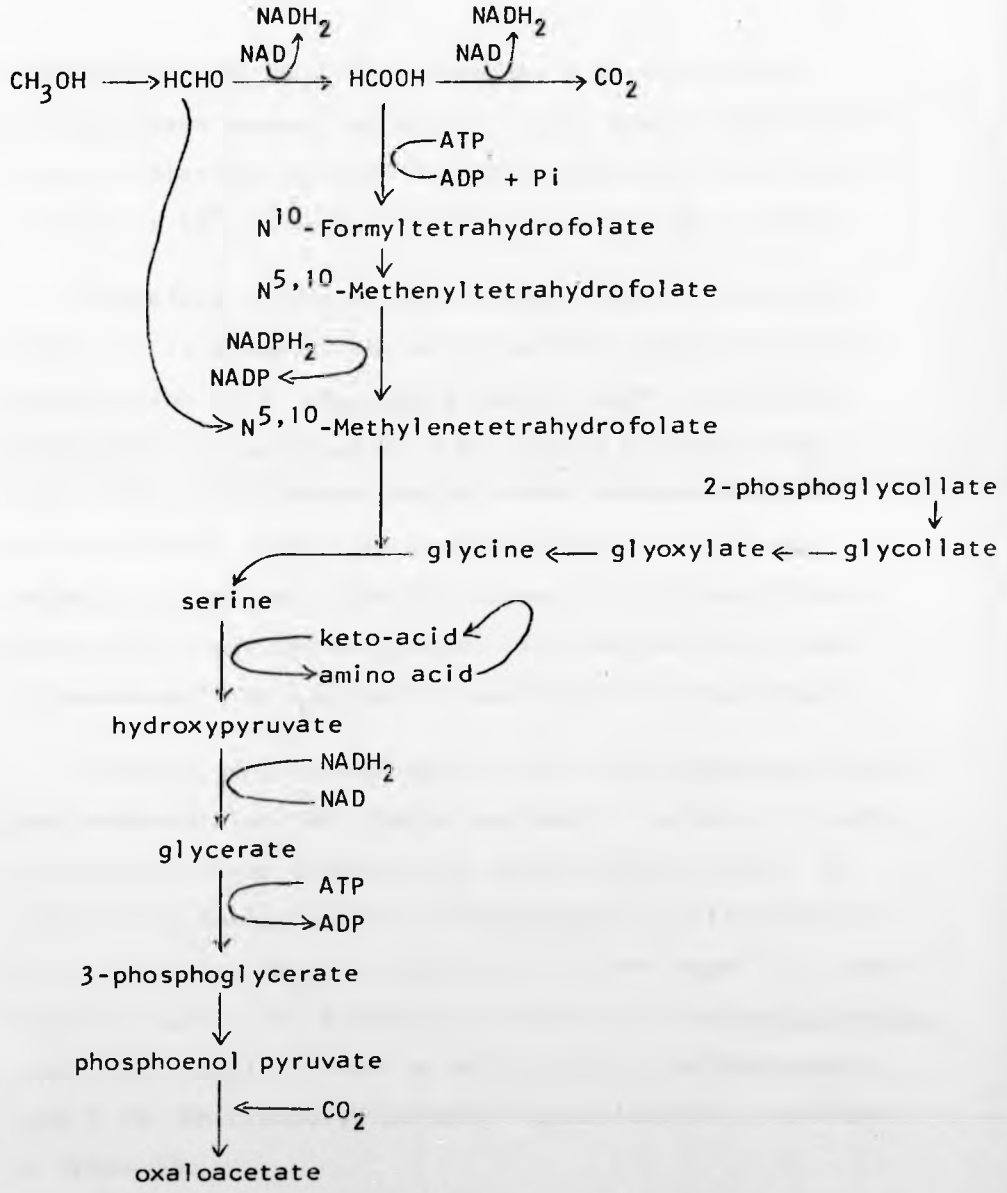


Fig. 63 Possible synthesis of C_4 skeleton from C_1 units and 2-phosphoglycollate (after Large and Quayle 1963)

45°C may suggest a greater need for a serine pathway of glycollate metabolism at the higher growth temperature. This correlates with the increased labelling of serine and glycine at 45°C observed by Whittenbury et al. (1976).

Therefore, these results suggest that although the RMP cycle is probably the only complete cycle for carbon assimilation in M. capsulatus (Bath), RuBP carboxylase allows for carbon dioxide to be used as an additional carbon source to methane and a serine pathway enables the inevitably formed phosphoglycollate to be further metabolised and may provide a means of utilising formate as an additional carbon source. The regulation of such a system would be complex and worthy of detailed study.

Clearly, the carbon metabolism of M. capsulatus (Bath) and probably also the 'Foster and Davis' strain, is very different to that reported for other methylotrophs. A number of other properties of Methylococcus also suggest that this genus cannot be allied to either type I or type II methylotrophs. It is therefore suggested that Methylococcus should be classified with a third group of methylotrophs, type X (R. Whittenbury, personal communication), as shown in Table 25.

Table 25 Classification of obligate methylotrophs

	Type I (*Methylomonas* sp)	Type X (*Methylococcus* sp)	Type II (*Methylosinus*, *Methylocystis* sp)
Membrane arrangement	Bundles of vesicular discs	Bundles of vesicular discs	Paired membranes, layers around periphery
G + C (%)	50-54	62.5	62.5
TCA cycle	incomplete	incomplete	complete
Carbon assimilation pathway	RMP cycle	RMP cycle + serine pathway?	serine cycle
RuBP carboxylase	absent	present	absent

A number of areas for further study have been indicated in the text. However, one area that has not been discussed concerns the possible use of methylotrophs as a source of single cell protein of which M. capsulatus has been the subject of considerable interest. The presence of auxiliary carbon assimilation pathways in this organism suggest possible methods of increasing growth yields. The provision of carbon dioxide as an additional carbon source to methane may be one such method, and in this context, possible use may also be made of the ability of M. capsulatus to oxidise inorganic compounds which could possibly function as energy sources to support carbon dioxide fixation.

Alternatively, a carbon dioxide fixation system could be viewed as an energy drain in a situation where methane is not limiting such as in an industrial fermenter A search for strains of Methylococcus without RuBP carboxylase could therefore be worthwhile.

11. EVOLUTIONARY ASPECTS OF THE RuBP CARBOXYLASE FROM METHYLOCOCCUS CAPSULATUS AND RHODOMICROBIUM VANNIELII (RM5)

The work presented above prompts discussion on two aspects of the evolution of autotrophy. Firstly, consideration must be given to the evolution of RuBP carboxylase in light of the elucidation of the structure of this enzyme in R. vannielii (RM5) and M. capsulatus (Bath). Secondly, the presence of RuBP carboxylase and phosphoribulokinase in M. capsulatus provides the basis for an important relationship between methylotrophs and those bacteria assimilating carbon dioxide via a Calvin cycle.

Most evolutionary arrangements of autotrophs place organisms in the order shown in Table 1. This ordering is based upon a comparison of metabolic capabilities and is paralleled by trends in increasing molecular size, quaternary structure and active site architecture of RuBP carboxylase. Such analysis has led to the postulated scheme for evolution of RuBP carboxylase shown in Figure 11 (McFadden and Tabita, 1974; McFadden, 1975). The evolution of this enzyme can also be correlated to the apparent morphogenetic evolution expressed by the Rhodospirillaceae (Fig. 12). The relatively high molecular weight (430,000), L_6S_6 subunit structure and inhibition by 6-phosphogluconate

of the R. vannielii (RM5) RuBP carboxylase in comparison to the low molecular weight (114,000), L_2 subunit structure and non-inhibition by 6-phosphogluconate, of the R. rubrum enzyme (Tabita and McFadden, 1974a), is in accordance with the greater cell-cycle complexity of R. vannielii (RM5). Although needing confirmation, the possible existence of both low molecular weight (360,000; L_6) and high molecular weight (550,000; L_8S_8) forms of RuBP carboxylase in R. spheroides and R. capsulata (Gibson and Tabita, 1977a, b) correlates with the intermediate degree of cell-cycle complexity in these organisms. Furthermore, sucrose density gradient centrifugation of the RuBP carboxylase from a newly isolated Rhodopseudomonas sp., akin to R. palustris, suggests that the enzyme has a molecular weight of approximately 300,000 (K. Eckersley, University of Warwick, personal communication), which again correlates with the morphogenetic scheme of evolution.

Doubts have already been expressed in the General Introduction regarding the quaternary structure assigned to RuBP carboxylases allegedly comprising only large subunits. These doubts have been augmented by the apparently unstable structure of the R. vannielii (RM5) enzyme. Consequently, correlation of quaternary structure with RuBP carboxylase evolution must be approached warily. Even so, it is of

interest to speculate as to the time of emergence of RuBP carboxylase with both large and small subunits. The L_6S_6 structure of the R. vannielii (RM5) enzyme suggests that the acquisition of small subunits pre-empted the development of the L_8S_8 structure characteristic of higher plants, algae and most prokaryotes. It may therefore be particularly worthwhile to examine a wider range of Rhodospirllaceae to further clarify this situation. However, the L_6S_6 subunit structure of the RuBP carboxylase from M. capsulatus (Bath) and P. oxalaticus (McFadden, 1977) indicates that this line of evolution was not confined to the photosynthetic organisms.

The molecular weight (360,000), subunit structure (L_6S_6) and inhibition by 6-phosphogluconate of the M. capsulatus (Bath) RuBP carboxylase, suggests that this organism was neither an early nor late developer on the time scale of autotrophic evolution. How then does this fit with current ideas on the evolution of autotrophy?

There is considerable indirect evidence, summarised in the General Introduction, to suggest that the Calvin cycle has evolved from the RMP cycle (Quayle and Ferenci, 1978). In view of the adaptability of bacteria and the many convergences and divergences that must have occurred during the 3 to 4 thousand million years that autotrophy has been evolving, it

is doubtful whether a present day organism exists demonstrating a metabolism which may be considered to be a precursor of the Calvin cycle. It is however, tempting to suggest that M. capsulatus, with a RMP cycle RuBP carboxylase and phosphoribulokinase, is such an organism. However, were M. capsulatus a transition organism between RMP and Calvin cycles, a primitive form of RuBP carboxylase would have been expected to be present, possibly with alternative carboxylase activity. Although a primative form of enzyme was not found, it would be of interest to probe this RuBP carboxylase for activity towards other substrates and for competitive inhibitors, such as phosphoenolpyruvate, which may suggest a previous function of the enzyme. The inference of Reh et al., (1977) that the genes for RuBP carboxylase and phosphoribulokinase are carried on a plasmid(s) in Ps. facilis, suggests an alternative mechanism by which these enzyme activities may have arisen in M. capsulatus (Bath).

The intermediate molecular weight of the M. capsulatus (Bath) RuBP carboxylase and the existence of a pathway for 2-phosphoglycollate metabolism, suggests that this organism may well have been a carbon dioxide assimilating autotroph which gained the ability to utilise methane. Within its natural environment, the utilisation of methane and/or carbon dioxide may give the organism a selective advantage

and consequently both modes of carbon assimilation have been retained. In this respect, the metabolism of M. capsulatus (Bath) may be viewed as an extension of the metabolism shown by a number of organisms (e.g. Paracoccus denitrificans, P. oxalaticus) which assimilate reduced C_1 compounds (methanol, formate) as carbon dioxide via a Calvin cycle. These organisms are probably carbon dioxide fixing autotrophs by design that have gained the ability to oxidise other C_1 compounds and so obtain greater nutritional versitility (Quayle and Ferenci, 1978) In addition to enzymes for methane oxidation, M. capsulatus may have also acquired additional biosynthetic enzymes and in this respect, it would be of interest to examine the substrate specificity and kinetics of the hexulose phosphate synthese and hexose phosphate isomerase from this organism, in order to elucidate possible 'precursor' enzymes.

Although it is not suggested that the RMP cycle in all methylotrophs has evolved from the Calvin cycle, the situation in M. capsulatus (Bath) is one that promotes speculation as to the origin of this unique organism. Little discussion has been focussed on the derivation of the serine pathway for C_1 assimilation. However, evidence from radiotracer experiments presented both in this thesis and by Whittenbury et al. (1976), suggests that certain reactions of the serine pathway may function in M. capsulatus

(Bath) in the metabolism of 2-phosphoglycollate. The inference to be made here, is that the serine pathway may have evolved as a result of the oxygenase activity of RuBP carboxylase.

Although its precise nature is unclear, the results obtained from M. capsulatus (Bath) do indicate an inter-relationship between methane assimilation by the RMP cycle and carbon dioxide fixation by the Calvin cycle. With the many other close morphological and biochemical relationships between the methane oxidisers and bacteria assimilating carbon dioxide, indicated in the General Introduction and by Whittenbury and Kelly (1977), there is a clear case to be made for bringing all these C_1 utilisers together under the term autotrophs.

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EVIDENCE FOR THE PRESENCE OF RIBULOSE 1,5-BISPHOSPHATE CARBOXYLASE AND PHOSPHORIBONUCLEASE IN *METHYLOCOCCUS CAPSULATUS* (BATH)

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1. Introduction

The methylotrophic bacteria are not known to be capable of utilising CO_2 as the sole source of carbon for growth. Assimilation of CO_2, however, does occur both in Type I and Type II organisms [1], but the reductive pentose cycle for CO_2 fixation has not been implicated in either case.

This study presents evidence for the presence of the key enzymes of the reductive pentose cycle, ribulose-1,5-bisphosphate carboxylase and phosphoribulokinase in extracts of *Methylococcus capsulatus* (Bath).

2. Materials and Methods

2.1. Growth of bacteria and preparation of extracts

M. capsulatus strain Bath [3] was grown at 45°C in batch culture on ammonium plus mineral salts medium [4]. Soluble and particulate fractions from crude cell extracts were prepared as described by Colby and Dalton [5].

2.2. Cell-free CO_2 fixation

CO_2 fixation was measured at 45°C in a reaction mixture (0.25 ml final volume) which contained: 15 μmol Tris–HCl, pH 8.2; 2.5 μmol of $MgCl_2$, 5 μmol of $NaH^{14}CO_3$ (specific activity 0.8 μCi/μmol unless stated otherwise); soluble extract (0.4 mg of protein); when required, NADH and ATP (0.2 μmol of each). After preincubation for 5 min, the reaction was started by the addition of 0.2 μmol of the test substrate. The reaction was stopped after a further 5 min by the addition of 100 μl of 12 M formic acid. A 200 μl sample of the final reaction mixture was evaporated to dryness in a scintillation vial, resuspended in 1 ml of water and radioactivity (fixed ^{14}C) measured in a liquid scintillation spectrometer.

2.3. Assay for 3-hexulose phosphate synthase

Enzyme activity was measured by the incorporation of label from [^{14}C]formaldehyde into sugar phosphates with D-ribose 5-phosphate as substrate [6]. Assays were performed at 45°C.

2.4. Chromatography

Radioactive compounds formed during the cell-free CO_2 fixation assays were analysed by two-dimensional ascending paper chromatography, and autoradiography. The solvent system was phenol : water : glacial acetic acid : ethylenediamine-tetraacetic acid (1 M) (840 : 149 : 10 : 1), in the first direction, and equal volumes of propionic acid : water (620 : 790) and *n*-butanol : water (1246 : 84) in the second direction [7]. Products were identified by co-chromatography with carrier organic compounds. 3-phosphoglyceric acid was detected with a molybdate reagent [8].

2.5. Enzyme purification

Ribulose 1,5-bisphosphate carboxylase was partially purified by centrifugation into a 0.2 M to 0.8 M discontinuous sucrose gradient [9].

TABLE 1

The fixation of carbon dioxide by cell-free soluble extracts of *Methylococcus capsulatus* (Bath).

Test substrate	CO_2 fixation (nmol CO_2 fixed · min^{-1} mg $protein^{-1}$)
None	0.2
Ribulose 1,5-P_2	9.4
Ribulose 1,5-P_2 (boiled extract)	0.3
Ribulose 1,5-P_2 + ATP	10.1
Ribulose 1,5-P_2 + NADH	9.0
Ribulose 1,5-P_2 + ATP + NADH	9.0
Ribulose 5-P	0.4
Ribulose 5-P + ATP	3.3
Ribulose 5-P + NADH	0.5
Ribulose 5-P + ATP + NADH	2.4
Ribose 5-P	0.2
Ribose 5-P + ATP	2.5
Ribose 5-P + NADH	0.3
Ribose 5-P + ATP + NADH	1.8

3. Results

Ribulose 1,5-bisphosphate and its immediate precursors in the reductive pentose cycle, were tested for their ability to act as carboxylation substrates for enzymes present in soluble, cell-free extracts of *M. capsulatus* (Bath). Activation of CO_2 fixation by ATP and NADH, as seen in *Nitrobacter winogradskyi* [10], was also investigated (Table 1).

Ribulose 1,5-bisphosphate stimulated CO_2 fixation whereas ribulose 5-phosphate and ribose 5-phosphate both required ATP for activity. This ATP requirement suggests that ribulose 5-phosphate and ribose 5-phosphate must be converted to ribulose, 1,5-bisphosphate, via a phosphoribulokinase, before CO_2 fixation can occur. No requirement was observed for NADH.

To determine the products formed by the ribulose 1,5-bisphosphate stimulated CO_2 fixation, samples from reaction mixtures containing partially purified enzyme and $NaH^{14}CO_3$ at a specific activity of 8 μCi/μmol, were analysed by two-dimensional chromatography. A single radioactive spot was detected which co-chromatographed with 3-phosphoglyceric acid, the product of ribulose 1,5-bisphosphate carboxylase.

Both soluble and particulate fractions were tested for 3-hexulose phosphate synthase activity to determine whether ribulose 1,5-bisphosphate stimulated CO_2 fixation, was mediated by this enzyme (Table 2).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis of the partially purified ribulose 1,5-bisphosphate carboxylase, indicated that it is composed of both large and small subunits as are plant, and the

TABLE 2

The activities of 3-hexulose phosphate synthase and ribulose 1,5-bisphosphate carboxylase in soluble and particulate fractions from crude cell-free extracts of *Methylococcus capsulatus* (Bath).

Fraction	3-Hexulose phosphate synthase (nmol substrate fixed · min^{-1} mg $protein^{-1}$)	Ribulose 1,5-bisphosphate carboxylase (nmol CO_2 fixed · min^{-1} mg $protein^{-1}$)
Soluble	12	10.5
Particulate	306	2.9

de by cell-free soluble extracts of *Methylococcus capsulatus* (Bath).

	CO_2 fixation (nmol CO_2 fixed · min^{-1} mg $protein^{-1}$)
	0.2
	9.4
act)	0.3
	10.1
	9.0
DH	9.0
	0.4
	3.3
	0.5
l	2.4
	0.2
	2.5
	0.3
	1.8

phate and its immediate pre-
pentose cycle, were tested for
boxylation substrates for
le, cell-free extracts of
tivation of CO_2 fixation by
in *Nitrobacter winogradskyi*
ed (Table 1).
phate stimulated CO_2 fixation
phate and ribose 5-phosphate
ctivity. This ATP require-
ose 5-phosphate and ribose
nverted to ribulose, 1,5-bis-
oribulokinase, before CO_2
equirement was observed for

To determine the products formed by the ribulose 1,5-bisphosphate stimulated CO_2 fixation, samples from reaction mixtures containing partially purified enzyme and $NaH^{14}CO_3$ at a specific activity of 8 μCi/μmol, were analysed by two-dimensional chromatography. A single radioactive spot was detected which co-chromatographed with 3-phosphoglyceric acid, the product of ribulose 1,5-bisphosphate carboxylase.

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phosphate synthase and ribulose 1,5-bisphosphate carboxylase in soluble and particulate fractions
s of *Methylococcus capsulatus* (Bath).

ulose phosphate synthase substrate fixed · min^{-1} mg $protein^{-1}$)	Ribulose 1,5-bisphosphate carboxylase (nmol CO_2 fixed · min^{-1} mg $protein^{-1}$)
	10.5
	2.9

majority of microbial, ribulose 1,5-bisphosphate carboxylases [11].

4. Discussion

Evidence presented in this paper demonstrates the existence of ribulose 1,5-bisphosphate carboxylase and phosphoribulokinase activity in *M. capsulatus* (Bath). To the best of my knowledge, this is the first report which suggests that the reductive pentose cycle may operate in a species of methane oxidizing bacteria.

Although the specific activity of CO_2 fixation in crude extracts is considerably lower than that observed in autotrophically grown *Nitrobacter* [10], it should be realized that Methylococcus derives most of its fixed carbon from the oxidation of methane without the intermediate formation of carbon dioxide; CO_2 fixation only represents a small proportion of the total carbon assimilated by the cell. Furthermore, the value observed for the ribulose 1,5-bisphosphate carboxylase activity in *M. capsulatus* (Bath) is comparable with that observed in crude extracts of *Rhodospirillum rubrum* [12] and *Rhodomicrobium vannielli* (unpublished results) when grown heterotrophically.

The comparatively low activities for the carboxylase, as measured with ribulose 5-phosphate or ribose 5-phosphate as substrate, and with ATP, suggests that the kinase, which would be necessary to convert the pentose mono-phosphates to the bisphosphate, is present either in low concentration, or has low activity, or requires specific activation. The reason for the apparent decrease in activity in the presence of NADH, and with all the substrates, is at present unclear.

M. capsulatus (Bath) assimilates the bulk of its carbon from methane via 3-hexulose phosphate synthase. This enzyme catalyses the condensation of a C1 unit (formaldehyde) with a C5 acceptor (ribulose 5-phosphate). The possibility that 3-hexulose phosphate synthase assumes the role of a carboxylase in this organism was discounted because its activity was almost entirely associated with the particulate fraction in crude extracts. The carboxylase activity was found almost exclusively in the soluble fraction (Table 2).

Whether the reductive pentose cycle for CO_2 fixation operates in vivo in *M. capsulatus* (Bath) and in other methane oxidizers, has yet to be determined. A reappraisal of theoretical cell yield studies for growth on methane [13] may however be required.

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